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14. ABSTRACT
Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in the United States. The goal of this training grant is to develop urinary makers for prostate cancer detection and prognostication and to train two physicians in clinical research. In this year, we continue to evaluate the feasibility of detection of prostate cancer by molecular urinalysis. We have found HGF along with IL18Bpa were most increased in the prostatic fluids of patients with extensive disease compared to those with minimal disease. IL17, GITR, and ICAM-1 were elevated in prostatic fluid specimens with significant neutrophilic inflammation into gland lumina, and IL18Bpa, IL17, GITR, and ICAM-1 were elevated in specimens with significant lymphocytic inflammation in prostatic stroma. These prostatic fluid cytokines may be useful for early cancer detection and prognostication efforts and for assessment of prostatic inflammation, particularly if they can be found not only in prostatic fluids obtained ex vivo, but in expressed prostatic secretions or urine samples from men with prostates still in situ. In this direction, we have pursued the biology and relevance of two cytokines we found in prostate cancer secretions, endoglin and IL-18Bpa. Two manuscripts pertaining to these markers have been generated, one accepted and the other submitted for publication. In addition, we have continued to refine molecular urine cytology for the diagnosis of prostate cancer, with a manuscript in preparation.

15. SUBJECT TERMS
Prostate cancer, detection, urine, molecular analysis, urinalysis

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INTRODUCTION:

Serum prostate-specific antigen (PSA) and digital rectal examination (DRE) remain the standard of care for prostate cancer screening despite their limited ability to detect occult prostate cancer. It is estimated that 15% of men with a normal PSA and DRE harbor prostate cancer. The rate of false negative prostate biopsies is estimated to be between 20-35%. Clearly, more specific and sensitive tests are needed to spare unnecessary biopsies and better identify and prognosticate affected men with prostate cancer. The scope of this research is to study, develop, and optimize biomarkers for the detection and prognostication of prostate cancer by molecular urinalysis that may help discriminate benign from malignant conditions of the prostate.

BODY:

We continue to collect urine specimens for biomarker analysis. Optimized methods of urine collection and storage for prostate-specific biomarkers have been achieved. Routine collection of initial urine post-DRE and post-prostate biopsy are processed to various fractions for cells, protein and DNA. The urine sediment is the most active fraction for our DNA, specific protein, and cellular analyses. Supernatants or whole urine are used for cytokine assays.
This year our goals were 1) to comprehensively assess the protein profile of prostatic secretions, such that biomarkers found associated with aggressive prostate cancers and inflammation might be sought in voided urine, 2) to explore the markers endoglin and IL-18Bpa in the urine of prostate cancer patients and 3) to continue to optimize and study the ability of fluorescent molecular urine cytology to diagnose prostate cancer in voided urine samples after digital rectal examination (DRE).

Protein analysis: Prostatic secretions from 40 radical prostatectomy specimens were assessed by cytokine antibody array, and then most upregulated proteins associated with aggressive prostate cancers were quantitated by ELISA. This work resulted in a publication (Fujita et al, Appendix 1) and in our pursuing several interesting biomarkers involved in cancer and inflammation more thoroughly. Our publication suggests that locally present molecules such as HGF and IL18Bpa are associated with large volume prostate cancer (Appendix 1, Figure 1) and that certain cytokines found within prostatic fluid are associated with discrete forms of inflammatory responses (Appendix 1, Figure 3, 4, and 5). Urinary endoglin was also found to be elevated in a separate study involving patients with prostate cancer (Appendix 2, Figure 1 and 2). In our cohort of patients at increased risk of prostate cancer, urinary endoglin performed better than PSA (Appendix 2, Figure 3), and we studied its sensitivity and specificity in detection prostate cancer (Appendix 2, Table 2). Interestingly, serum endoglin levels were similar in normal patients and patients with known prostate cancer. However, in patients with prostate cancer, elevated endoglin levels were associated with non-organ confined prostate cancer (Appendix 2, Figure 4 and 5).
Our investigations have also led us to evaluate IL-18Bpa as a novel urinary marker for prostate cancer. We have summarized our current data in a submitted manuscript (Appendix 3). We found that IL18Bpa was expressed and secreted by the prostate cancer cell lines DU145 and PC3, but not by LNCaP and CWR22, upon interferon-gamma stimulation (Appendix 3, Figure 3a). The IL18Bpa secreted from DU145 and PC3 functionally inhibited IL18 (Appendix 3, Figure 3c). Conditioned medium from IL18Bpa-overexpressed PC3 cells suppressed CD8+ IFN-gamma+ cells and TH1cells in human peripheral blood (Appendix 3, Figure 6). Immunohistochemical analyses showed positive IL18Bpa staining in prostate cancer cells as well as in macrophages in radical prostatectomy specimens (Appendix 3, Figure 5). Significant differences in post-DRE urinary IL18Bpa levels (normalized by total protein) were found between cases with and without cancer on biopsy ($p=0.02$) and serum IL18Bpa levels correlated with Gleason score ($p=0.03$) (Appendix 3, Figure 7). Our finding of elevated IL18Bpa secretion from prostate cancer cells suggests an attempt by cancer to escape immune surveillance, which we plan to continue to pursue.

Our studies with AMACR, AGR-2 and MYO-6 by Western blot have demonstrated that while they are present in post-DRE urine sediments, their levels appear to be quite low. We have been unable to achieve accurate quantitation of these proteins (ELISA or comparable assays), and our data have not shown these molecules to be tightly associated with cancer. They are upregulated in PIN and so may not prove as valuable as initially hoped. On the other hand, AMACR-specific antibodies have proven quite helpful in our molecular cytology work, as they stain cells suspected of being of prostatic origin and that may be cancerous or pre-cancerous. While on its own AMACR-staining is not diagnostic of prostate cancer, in combination with other cellular and architectural features of prostate cancer this marker is in widespread use diagnostically.

Cellular analysis: We have optimized a 4-marker fluorescent probe-set for the detection of intact prostate cancer cells in the voided urine of men suspected of having prostate cancer. We collect the urine after an extended DRE and immediately fix it for cytologic preparation. Compared with post-DRE conventional urine cytology for prostate cancer detection, which has proven highly specific but extremely insensitive, FISH analyses using these prostate specific markers have more than doubled its sensitivity. We are currently analysis this data and look forward to presenting it next year.

Training: Both Dr. Pavlovich and Dr. Chan have been active in mentored study with Drs. Isaacs and Trock. Bi-monthly lab meetings are standard. Both trainees have completed the Course of Research Ethics. Dr. Pavlovich successfully completed a biostatistics course at the Bloomberg School of Public Health. Both are physicians are pursuing a set of courses entitled the Science of Clinical investigation.

KEY RESEARCH ACCOMPLISHMENTS

1) Characterization of the cytokine profile of cancerous prostatic fluids and correlation with cancer and inflammation status. Identification of specific cytokine markers of prostatic inflammation, which can differentiate the types of inflammatory cells in the prostate (Appendix 1)

2) Identification of several novel biomarkers of prostate cancer, including IL18BPa and Endoglin, which hold particular promise as non-invasive urinary biomarkers with utility in prostate cancer diagnosis and prognosis (Appendices 2 and 3).

REPORTABLE OUTCOMES:

Manuscripts:

1) Fujita K et al. Cytokine profiling of prostatic fluid from cancerous prostate glands identifies cytokines associated with extent of tumor and inflammation. Prostate. 2008 Jun 1;68(8):872-82.

2) Fujita K et al. Endoglin (CD105) as a Urinary and Serum Marker of Prostate Cancer. Manuscript Manuscript in press, Int J Cancer (Appendix 2).

3) Fujita K et al. IL18 binding protein is produced by prostate cancer cells and its levels in urine and serum correlate with tumor status. Manuscript submitted. (Appendix 3).

Abstracts:

1) Fujita K et al. Endoglin as a potential urinary marker for prostate cancer detection. AUA 2008: Abstract No. 2091.

2) Fujita K et al. "Molecular cytology for prostate cancer detection: Multiplex fluorescent staining of urine sediment in the era of new prostate-specific biomarkers" AACR 2008: Abstract No. 3644

3) Fujita K et al. Molecular urine cytology for prostate cancer detection. ASCO 2008 Genitourinary Cancers Symposium - Abstract - No. 273

4) Fujita K et al. Endoglin (CD105) as a potential urinary marker for prostate cancer detection. ASCO 2008 Genitourinary Cancers Symposium - Abstract - No. 54

CONCLUSION:

Detection of prostate cancer by molecular urinalysis is feasible. We have found several interesting proteins to be upregulated in advanced prostate cancers (endoglin and IL18BPa) and have started to explore their biology (IL18BPa). We continue to address our aims of collecting and banking post-DRE urine samples for subsequent analysis of these and other biomarkers as they become available. In addition, our attempts to develop a urinary cytologic set of markers for prostate cancer cells shed in urine continue; we will hopefully be able to report on this in the near future. Ultimately, the goal is to compare and contrast various modalities of molecular urinalysis for prostate cancer, from protein to cellular to perhaps nucleic acid-level analyses, in the hopes of adding to the clinical utility and shortcomings of serum-based PSA for prostate cancer detection and prognostication.

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APPENDICES:

1) Fujita K et al. Cytokine profiling of prostatic fluid from cancerous prostate glands identifies cytokines associated with extent of tumor and inflammation. Prostate 2008, 68:872-882.

2) Fujita K et al. Endoglin (CD105) as a Urinary and Serum Marker of Prostate Cancer. Manuscript in press, *Int J Cancer*.

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Cytokine Profiling of Prostatic Fluid From Cancerous Prostate Glands Identifies Cytokines Associated With Extent of Tumor and Inflammation

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BACKGROUND. Cytokines are key mediators of inflammation that may relate to prostate cancer initiation and progression, and that may be useful markers of prostatic neoplasia and related inflammation. In order to better understand the relationship between cytokines and prostate cancer, we profiled cytokines in prostatic fluids obtained from cancerous prostate glands and correlated them to both cancer status and inflammatory grade.
METHODS. Prostatic fluid was collected from fresh radical prostatectomy specimens and analyzed by cytokine antibody microarray. For comparison, cases were selected from patients with either minimal or extensive cancer volume on final pathology. Among the cytokines with the greatest difference between the tumor volume groups, eight had their levels quantitated by ELISA. In addition, the grade of prostatic inflammation by neutrophils, macrophages and lymphocytes was scored for each case and examined for correlations with cytokine levels.
RESULTS. Among 174 cytokines analyzed, HGF was the most increased (6.57-fold), and along with IL18Bpa was significantly elevated in patients with extensive disease compared to those with minimal disease. IL17, GITR, and ICAM-1 were elevated in specimens with significant neutrophilic inflammation into gland lumina, and IL18Bpa, IL17, GITR, and ICAM-1 were elevated in specimens with significant lymphocytic inflammation in prostatic stroma.
CONCLUSIONS. Prostatic fluid cytokines were identified that may be useful for early cancer detection and prognostication efforts and for assessment of prostatic inflammation, particularly if they can be found not only in prostatic fluids obtained ex vivo, but in expressed prostatic secretions or urine samples from men with prostates still in situ. *Prostate 68: 872–882, 2008.*

KEY WORDS: **cancer; inflammation; cytokine**

INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of cancer-related death in men over 40 years of age in the United States [1]. The etiology of prostate cancer is not well understood. Chronic infection and inflammation are causes of cancer in the stomach, liver and large intestine. Data from histopathological, molecular histopathological, epidemiological, and genetic epidemiological studies show that chronic inflammation might also be important in prostate carcinogenesis [2]. Proliferative inflammatory atrophy (PIA), where proliferative glandular

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K. Fujita and C.M. Ewing contributed equally to this work.

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epithelium with the morphological appearance of simple atrophy occurs in association with inflammation, is thought to be a possible precursor to prostate cancer [3]. Chronic and/or acute glandular inflammation is indeed observed in many radical prostatectomy specimens [4].

Cytokines are proteins that are expressed from immune, epithelial, and stromal cells, that can be excreted into the lumina of glands [5,6]. Cells communicate with each other by networks of interrelated cytokines. Cytokines are not only key mediators of inflammation, but may also play important roles in the initiation and progression of prostate cancer. While some cytokine analysis of prostatic fluid from expressed prostatic secretions has been performed [5,6], a comprehensive cataloguing of cytokines from the cancerous prostate has not been reported. Such a cytokine profile may provide further insight into the mechanisms of prostate cancer initiation and progression, and may facilitate the exploration of new markers of prostatic neoplasia and inflammation. If chemopreventive strategies aimed at reducing prostatic inflammation are implemented, noninvasive markers of this process would be useful. In this study, we describe the cytokine profile of prostatic fluids obtained from cancerous prostate glands and correlate it to both cancer status and inflammation grade.

MATERIALS AND METHODS

Collection of Samples

Prostatic fluids were collected by squeezing ex vivo prostate glands that were freshly obtained following radical prostatectomy for prostate cancer and collecting drops of fluid from the protruding apical urethral stump. The radical prostatectomy specimens were then submitted for routine formalin fixation, sectioning, and pathologic analysis as per standard protocol [7]. Prostate glands with either minimal prostate cancer (M, $n = 20$) or extensive prostate cancer (E, $n = 20$) as estimated by tumor volume were chosen for this study. Specimens with minute foci of a maximum tumor area of less than 15 mm^2 were assigned to the M group, and specimens with a maximum tumor area of more than 80 mm^2 were assigned to the E group. The prostatic fluids were kept at $-80^{\circ}C$ until the cytokine determination experiments. Approval was obtained from our Institutional Review Board before initiating the study and all patients provided written informed consent.

Cytokine Antibody Array

A Raybio^{TM} Human Cytokine Array kit (Raybiotech, Norcross, GA) including 174 cytokines was used per the manufacturer's recommendations. Briefly, membranes immobilized with capture antibodies were blocked with 5% bovine serum albumin/triethanolamine-buffered saline (TBS) for 1 hr. Membranes were then incubated with prostatic fluid samples [1 ml, in 10-fold dilution with TBS and Complete protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN)] for 2 hr at room temperature. After extensive washing with TBS/0.1% Tween 20 (3 times, 5 min each) and TBS (twice, 5 min each) to remove unbound cytokines, membranes were incubated with biotin-conjugated anticytokine antibodies. Membranes were washed and then incubated with horseradish peroxidase-conjugated streptavidin (2.5 pg/ml) for 1 hr at room temperature. Unbound materials were washed out with TBS/0.1% Tween 20 and TBS. Finally, the signals were detected by the enhanced chemiluminescence system, followed by additional washing. Spots were visualized using enhanced chemiluminescence (ECL plus Western Blotting System, Amersham Biosciences, Pittsburgh, PA). Membranes were exposed to Kodak X-Omat radiographic film for 1 min per image. Each film was scanned into TIFF Image files, and spots were digitized into densities with Gel-Pro-Analyzer (Media Cybernetics, Bethesda, MD). The densities were exported into Microsoft Excel, and the background intensity was subtracted prior to analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Eight cytokines in prostatic fluids were measured by ELISA. A human ELISA kit (Raybiotech) was used to detect hepatocyte growth factor (HGF), interleukin 12p70 (IL12), glucocorticoid-induced tumor necrosis factor receptor (GITR), intercellular adhesion molecule 1 (ICAM-1), and neurotrophin-3 (NT-3). A Quantikine human immunoassay kit (R&D Systems, Minneapolis, MN) was used to detect interleukin 17 (IL17), and epithelial-neutrophil activating peptide (ENA78). DuoSet ELISA development system (R&D Systems) was used to detect interleukin 18 binding protein a (IL18Bpa). Each cytokines was measured based on the manufacturer's recommendations. For examples, to measure HGF, IL12, GITR, ICAM-1, and NT-3 prostatic fluids were diluted accordingly. Samples were added (100 μl/well) in duplicate for incubation for 2.5 hr at room temperature. Biotinylated antibodies were subsequently added (100 μl/well) and incubated for 1 hr at room temperature. Incubation with streptavidin-horseradish-peroxidase (for 15 min) was followed by detection with 3,3 V,5,5 V-tetramethylbenzidine (TMB) for 30 min. The reaction was stopped by the addition of 1.5 M H_2SO_4. Plates were read using a wavelength of 450 nm on a microplate reader (PHERA star, BMG LABTECH, Durham, NC).

Histological Analysis

Hematoxylin and eosin stained sections were used to assess the inflammatory status of the prostate. For each case, two sections were chosen from right posterior, left posterior, right anterior, and left anterior prostate at apex and middle (eight sections total) and were examined by light microscopy for the presence of neutrophils, macrophages and lymphocytes. An inflammation grade of 1 for neutrophils or macrophages (low-grade inflammation) was assigned to specimens in which neutrophils or macrophages were observed only in prostatic gland lumina, with no epithelial disruption, or in which neutrophils were not observed at all. A grade of 2 (high-grade inflammation) was assigned to specimens in which the gland lumina were filled with immune cells and/or pus and more than 10 neutrophils or macrophages were found in the epithelial lining under 40× magnification, or in which these immune cells were found in the interstitium with associated epithelial destruction. A grade of 1 for lymphocytes was assigned to the specimens in which confluent sheets of inflammatory cells with nodule/follicle formation were observed focally or multifocally in the stroma (less than 50% of area), while a grade of 2 was assigned to specimens in which those were observed diffusely in the stroma (more than 50% of area) in at least one section [8].

Data Analysis and Statistics

Positive control signals on each membrane were used to normalize cytokine signal intensities from cytokine antibody arrays. Then, the data were normalized to PSA levels in each prostatic fluid sample to account for differential yields of fluid actually of prostatic origin. Total PSA levels in each prostatic fluid sample were measured by Hybritech PSA assay on the Beckman Coulter Access Immunoassay System (Beckman Coulter, Inc., Fullerton, CA). The normalized intensity value of cytokines in each group (M or E) was converted into the relative n-fold change between groups.

Data from ELISA in prostatic fluids were also normalized to the average PSA levels in each prostatic fluid sample. Data from prostatic fluids were analyzed as categorized by tumor volume (M and E), Gleason score (6 and $\geq$7), or inflammation grade (1 or 2).

Statistical analyses were done using GraphPad Prizm 4.0 for Windows. Mann–Whitney tests were used to analyze the difference of two categories. Chi-square tests were used to analyze the correlations between tumor volume and inflammation grade. Spearman's correlations were used to analyze the correlations of two cytokines and that of cytokines and tissue weights or age. Statistical significance was defined as a P-value <0.05.

RESULTS

Cytokine Profile of Prostatic Fluid by Cytokine Array

The normalized intensity values of cytokines from group E (extensive volume prostate cancer) were divided by those from group M (minimal volume prostate cancer) to calculate the relative n-fold change. The ranked cytokine profile of the relative n-fold change obtained by cytokine array is listed in Table I; for a comprehensive listing see Supplementary Table. Among 174 cytokines analyzed, HGF was the most increased cytokine in group E (6.57-fold).

Correlation of HGF and IL18BPa With Cancer Status by ELISA

Among the cytokines with the greatest difference between groups E and M, we selected eight cytokines for further study (HGF, IL18Bpa, ICAM-1, IL17, NT-3, IL12, GITR, and ENA78) and confirmed their levels in

TABLE I. Cytokine Profile of Prostatic Fluid

Cytokine	Ratio (Ext/Min)	Average signal of Ext group (SD)	Average signal of Min group (SD)
HGF	6.57	118.24 (164.50)	18 (14.90)
IL18Bpa	2.58	4.37 (6.67)	1.69 (2.60)
ICAM-1	2.41	53.34 (68.50)	22.15 (23.09)
IL17	2.34	1.74 (2.78)	0.74 (1.15)
NT3	2.32	1.79 (2.38)	0.77 (1.31)
IL12p70	2.32	4.41 (5.92)	1.90 (1.48)
GITR	1.99	3.80 (4.56)	1.91 (1.69)
ENA78	1.74	20.93 (28.61)	11.99 (18.71)

Complete cytokine profile shown in Supplementary Table.
Ext, extensive prostate cancer, Min, minimal prostate cancer.

prostatic fluids quantitatively by ELISA. Each of these cytokines was elevated in group E; the HGF and IL18Bpa elevations were statistically significant compared to group M (Fig. 1). In an analysis based on Gleason score, only IL18Bpa was significantly elevated in specimens with high Gleason grade ($\geq$7). Strong correlations were noted between some cytokines, especially between ICAM-1 and GITR (Spearman's correlation coefficient r = 0.820), ICAM-1 and ENA78 (r = 0.782), and NT3 and GITR (r = 0.782) (Table II). No correlation was found between each cytokine and specimen weight. Weak correlations were found

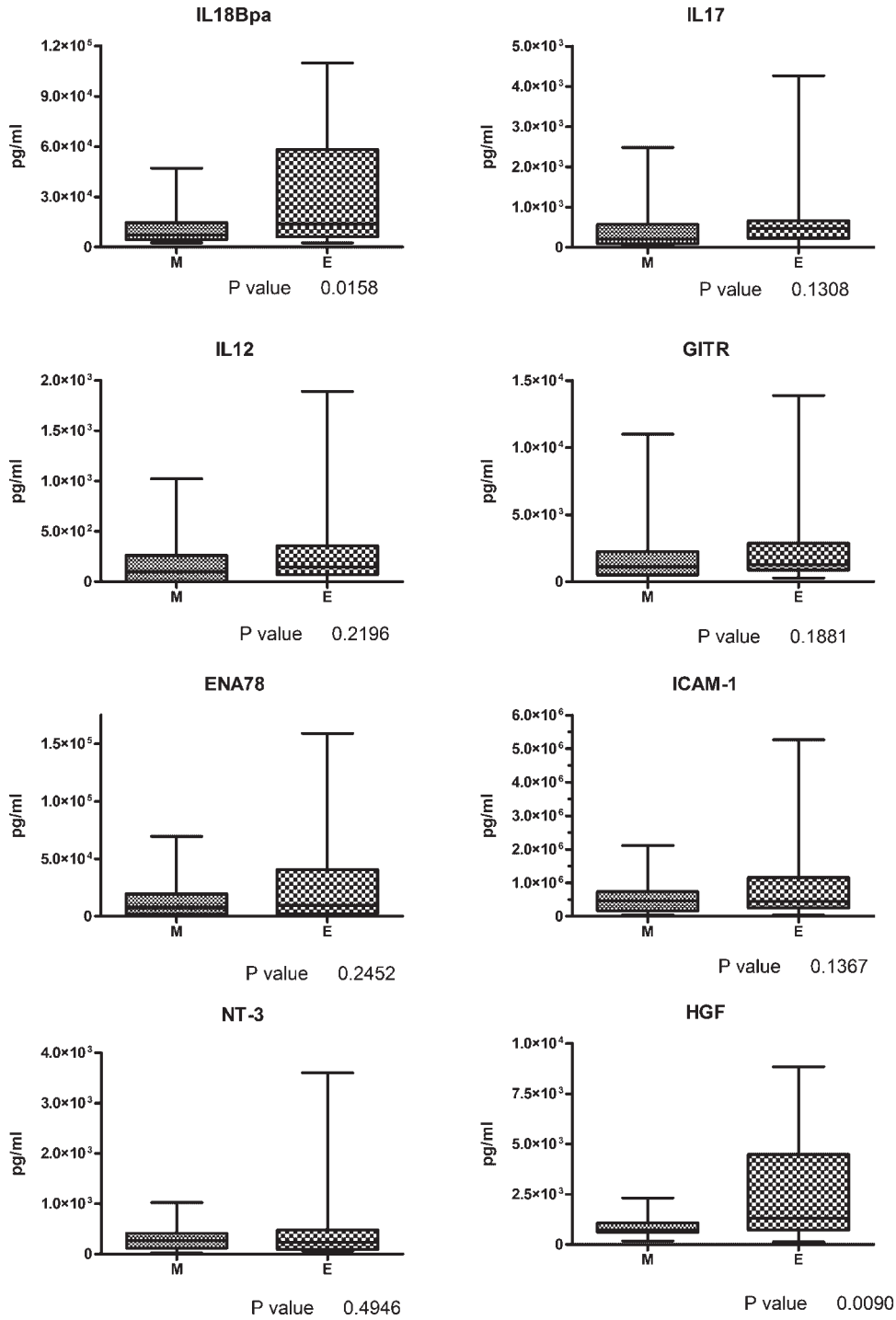

Fig. 1. Correlation of cytokines with cancer status. Each cytokine level measured by ELISA was analyzed stratified by cancer status. The HGF and IL18Bpa elevations of group E were statistically significant compared to group M [M: minimal prostate cancer (n = 20), E: extensive prostate cancer (n = 20)].

TABLE II. Correlations Between Eight Cytokines

	IL18Bpa	IL17	IL12p70	GITR	ENA78	ICAM-1	NT-3	HGF
IL18Bpa		0.2585 (0.1072)	0.2992 (0.0643)	0.5417 (0.0004)	0.339 (0.0324)	0.6246 (<0.0001)	0.4507 (0.0035)	0.3732 (0.0177)
IL17	0.2585 (0.1072)		0.6486 (<0.0001)	0.4986 (0.0012)	0.4026 (0.01)	0.4829 (0.0016)	0.4865 (0.0015)	0.2568 (0.1096)
IL12p70	0.2992 (0.0643)	0.6486 (<0.0001)		0.6175 (<0.0001)	0.5767 (0.0001)	0.5227 (0.0006)	0.5832 (<0.0001)	0.3227 (0.0451)
GITR	0.5417 (0.0004)	0.4986 (0.0012)	0.6175 (<0.0001)		0.6854 (<0.0001)	0.8203 (<0.0001)	0.7816 (<0.0001)	0.3967 (0.0124)
ENA78	0.339 (0.0324)	0.4026 (0.01)	0.5767 (0.0001)	0.6854 (<0.0001)		0.7822 (<0.0001)	0.5548 (0.0002)	0.3946 (0.0118)
ICAM-1	0.6246 (<0.0001)	0.4829 (0.0016)	0.5227 (0.0006)	0.8203 (<0.0001)	0.7822 (<0.0001)		0.6576 (<0.0001)	0.4927 (0.0012)
NT-3	0.4507 (0.0035)	0.4865 (0.0015)	0.5832 (<0.0001)	0.7816 (<0.0001)	0.5548 (0.0002)	0.6576 (<0.0001)		0.2874 (0.0721)
HGF	0.3732 (0.0177)	0.2568 (0.1096)	0.3227 (0.0451)	0.3967 (0.0124)	0.3946 (0.0118)	0.4927 (0.0012)	0.2874 (0.0721)	

The value listed is the Spearman's correlation coefficient, and that in parentheses is the *P*-value.

between increasing age and IL12 (r = 0.3480) and increasing age and NT3 (r = 0.4001).

Relationship Between Cytokine Levels and Prostatic Inflammation

Routine histochemical analysis demonstrated that neutrophils and macrophages were present in prostatic glandular lumina (grade 1 inflammation, Fig. 2) and in the lining of the prostate epithelium (grade 2 inflammation). Isolated lymphocytes aggregated in the stroma surrounding ducts (grade 1 cases) and lymphoid follicles were occasionally noted (grade 2). There was no statistical correlation between the inflammation grade by each immune cell type assessed (neutrophil, macrophage, and lymphocyte) and tumor volume (M and E) (Chi-square test). Data pertaining to eight cytokines found in prostatic fluids were analyzed according to inflammation grade in the radical prostatectomy specimens. In cases stratified by neutrophil inflammation, IL17, GITR, and ICAM-1 were significantly associated with increasing (grade 2) inflammation ($P < 0.05$), and ENA78 ($P = 0.0594$) and NT-3 ($P = 0.0554$) levels were close to reaching statistical significance (Fig. 3). In cases stratified by macrophage inflammation, none of these cytokines was significantly elevated in grade 2 versus grade 1 infiltrates (Fig. 4). In cases stratified by lymphocyte inflammation, IL18Bpa, IL17, GITR and ICAM-1 were significantly elevated in grade 2 lymphocytic infiltration ($P < 0.05$) (Fig. 5).

DISCUSSION

The prostate gland secretes many substances, including citric acid, polyamines, zinc, and cytokines. Cytokines are secreted from lymphocytes, macrophages, and mast cells, and also from prostatic epithelial and stromal cells [9–11]. Recently, cytokines have been shown to play important roles in prostatic inflammation, carcinogenesis, and cancer progression [9,12,13]. In this study, we for the first time describe the cytokine profile of prostatic fluid from cancerous prostates. A better knowledge of the cytokines present in prostate fluid may aid in understanding the implications of the prostatic cytokine network on inflammation, carcinogenesis, and prostate cancer progression, and may lead to novel cancer detection strategies.

We initially studied prostatic cytokines by array, and catalogued the most prevalent cytokines noted from fluids obtained from prostate specimens with extensive cancer as compared to those from prostates with minimal cancer. Among the most up-regulated cytokines in cases with extensive disease, we selected HGF, IL18Bpa, ICAM-1, IL17, NT-3, IL12, GITR, and ENA78, for more quantitative assessment by ELISA. These cytokines were selected from the groups of

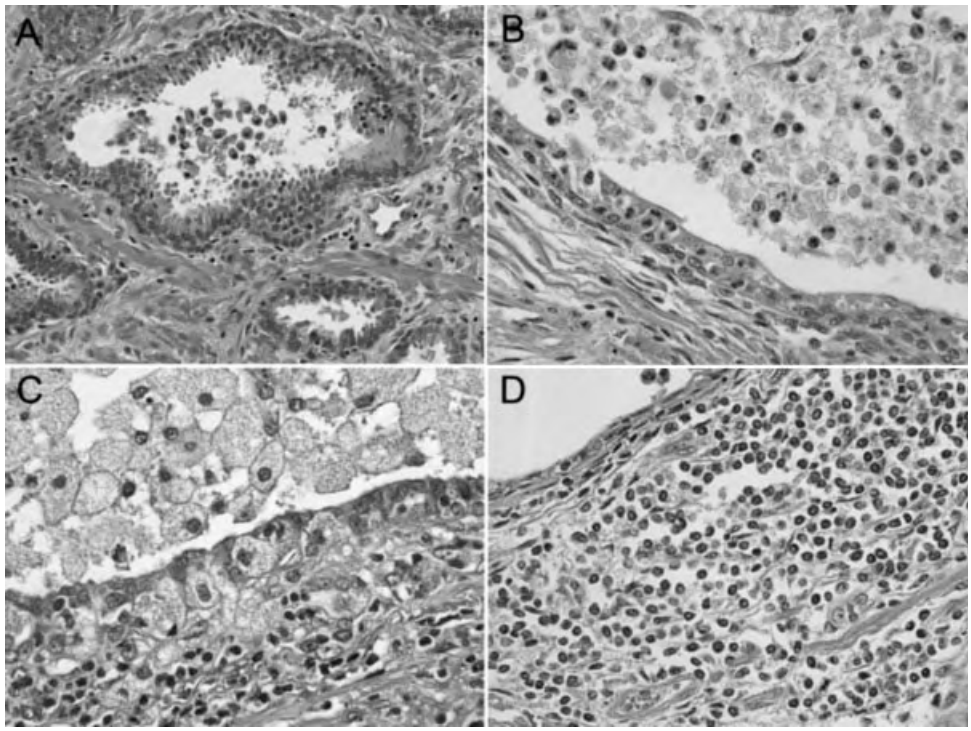

Fig. 2. Inflammation of prostate by neutrophils, macrophages or lymphocytes. **A**: Some neutrophils or macrophages were observed only in gland lumina, with no epithelial destruction. **B**: A gland lumen filled with neutrophils and pus, with neutrophils noted within the epithelial lining (inflammation grade 2). **C**: A gland lumen filled with macrophages and pus, with macrophages noted within the epithelial lining (inflammation grade 2). **D** In grade 2 lymphocytic inflammation, confluent sheets of inflammatory cells with nodule/follicle formation were observed diffusely in the stroma (more than 50% of the area) in at least one section.

cytokines that were elevated because of their known roles in cancer and inflammation-related pathways.

HGF has been shown to be important in prostate cancer progression, invasion and metastasis [14]. IL18Bpa and IL12 are involved in the Th1 immune response [15,16]. IL-12 is the major cytokine responsible for the differentiation of T helper 1 cells, which are in turn potent producers of IFN-γ [15]. IL18Bpa is a potent inhibitor of IL-18, which is a central player in inflammation and in the immune response, and which has antineoplastic properties. ICAM-1 is expressed by leukocytes, epithelial cells, endothelial cells and tumor, stimulates neovascularization [17], and is elevated in the serum of patients with cancer [18]. IL-17 is a pro-inflammatory cytokine, that plays a crucial role in the development of autoimmunity and allergic reactions, and the expression of IL17 from Th17 is stimulated by IL23, which promotes tumor incidence and growth [19]. NT-3 is a member of the neurotrophins; it is expressed by prostate epithelial cells and stromal cells from prostates with cancer, but not by benign prostatic tissue [10]. GITR is expressed by T regulatory cells (Treg) as well as activated T cells and NK cells. ENA-78 produced by monocytes, macrophages, fibroblasts, endothelial cells, and several types of epithelial cells is a member of the CXC family of chemokines, and acts as a potent chemoattractant and activator of neutrophil function as well as an angiogenic factor in cancer [20,21].

In the cytokine antibody array portion of our study, HGF in prostatic fluid was the cytokine most increased in extensive disease cases, a finding that was confirmed statistically by ELISA. HGF, which can be derived from a variety of tissues, is known to be elevated in the serum of men with metastatic prostate cancer [22]. In the prostate, stromal cells secrete HGF, which acts locally on prostate epithelial cells expressing its receptor, the tyrosine kinase c-Met. Prostate cancer can also express HGF via stimulation by IL-1β, PDGF, bFGF, VEGF, and EGF derived from stromal cells [23]. The intracellular cascade that ensues secondary to c-Met phosphorylation appears to be responsible for most of the effects of HGF, including its pro-mitogenic and antiapoptotic properties, and its effects on developmental cell migration. Alterations of HGF or c-Met levels can affect these and other biological pathways associated with cancer progression [14].

While prostatic fluid HGF and IL-18Bpa levels were related to tumor volume, prostatic fluid IL-18BPa, IL17, GITR, and ICAM-1 levels were correlated with inflammation. These results indicate that the above cytokines may be regulated or released by specific immune cells

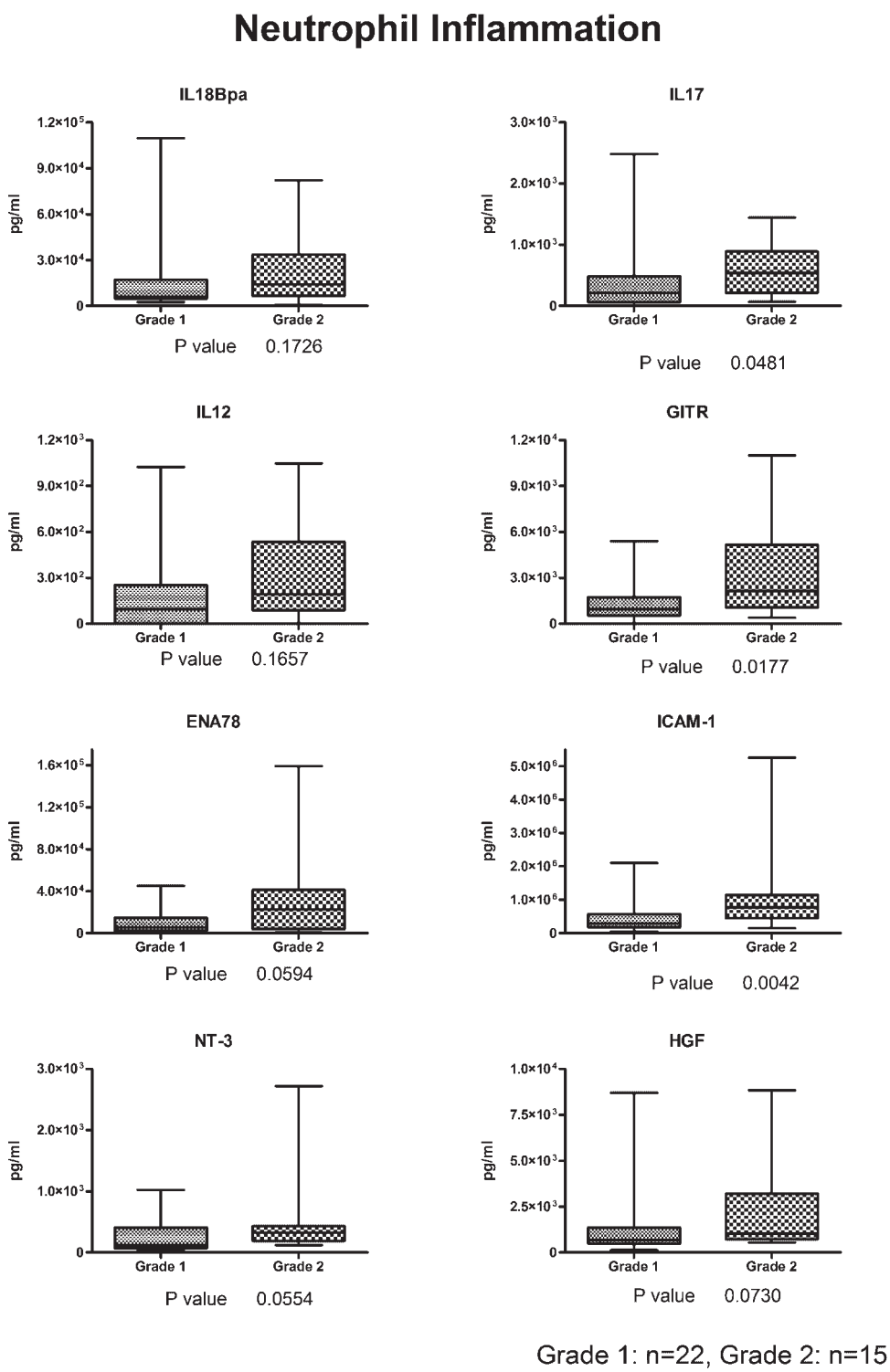

Fig. 3. Relationship between cytokine levels and neutrophil inflammation. ILI7, GITR, and ICAM-1 were significantly associated with grade 2 inflammation ($P < 0.05$).

in the gland lumina (neutrophils) or in the epithelial lining or stroma (lymphocytes). In fact, IL17 is expressed by Th17, a distinct T cell subset that stimulates the production of cytokines that attract neutrophils to the site of inflammation [24]. Neutrophils use ICAM-1 on epithelial cells to migrate across the epithelial lining [25], and ICAM-1 is also one of the cytokines induced by IL17 [24].

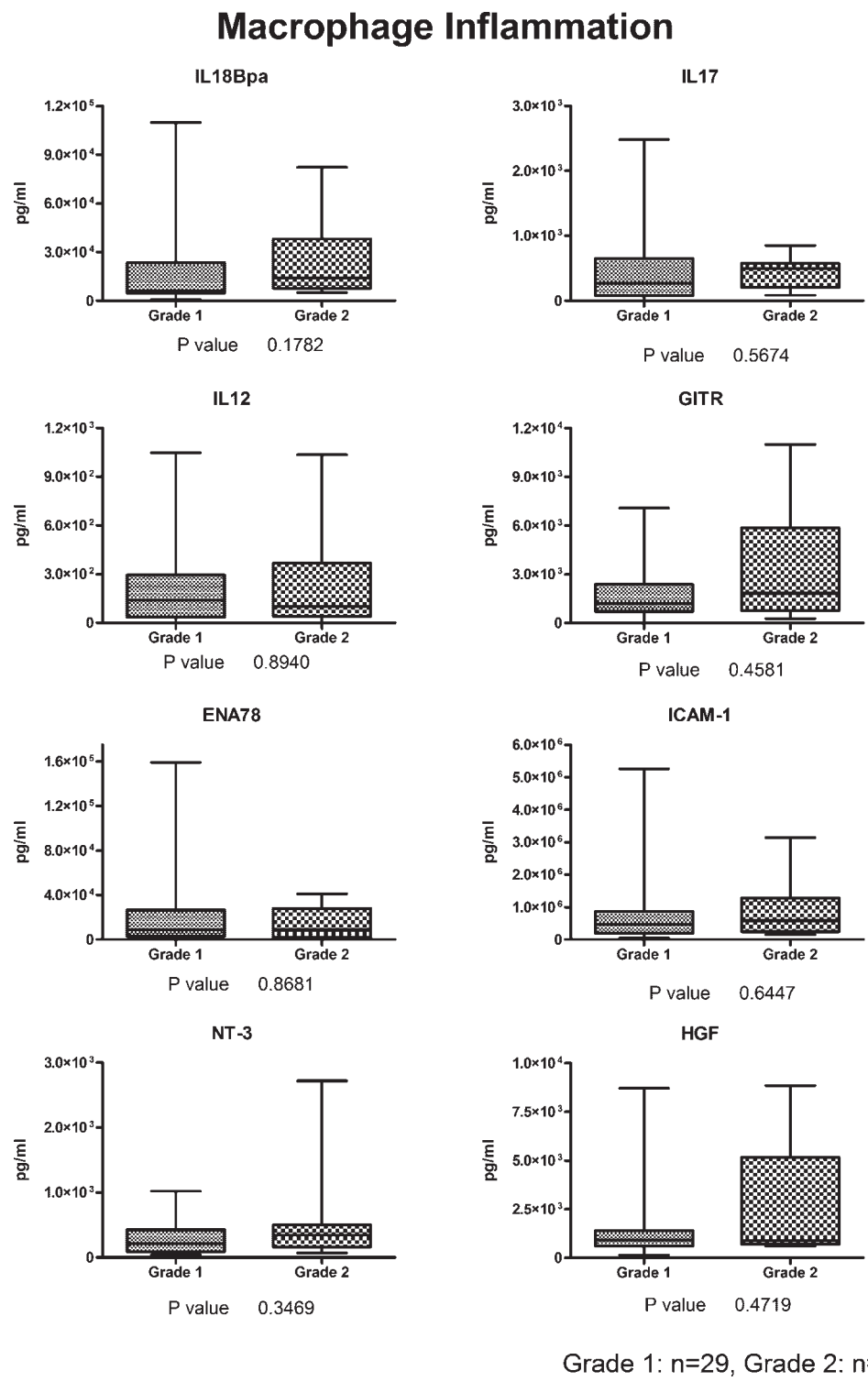

Fig. 4. Relationship between cytokine levels and macrophage inflammation. No cytokine was significantly elevated in grade 2 versus grade I inflammation.

Among these cytokines, IL18Bpa and GITR may be new markers of prostatic inflammation that is associated with cancer initiation or progression. Importantly, IL18Bpa was correlated with both cancer and inflammation status in our study.

IL18 plays an important role in host defenses against various infectious microbes, but overproduction of IL18 causes autoimmune diseases and inflammatory tissue damage [26]. The excretion of IL18Bpa from monocytes and NK cells is induced by IL12 and

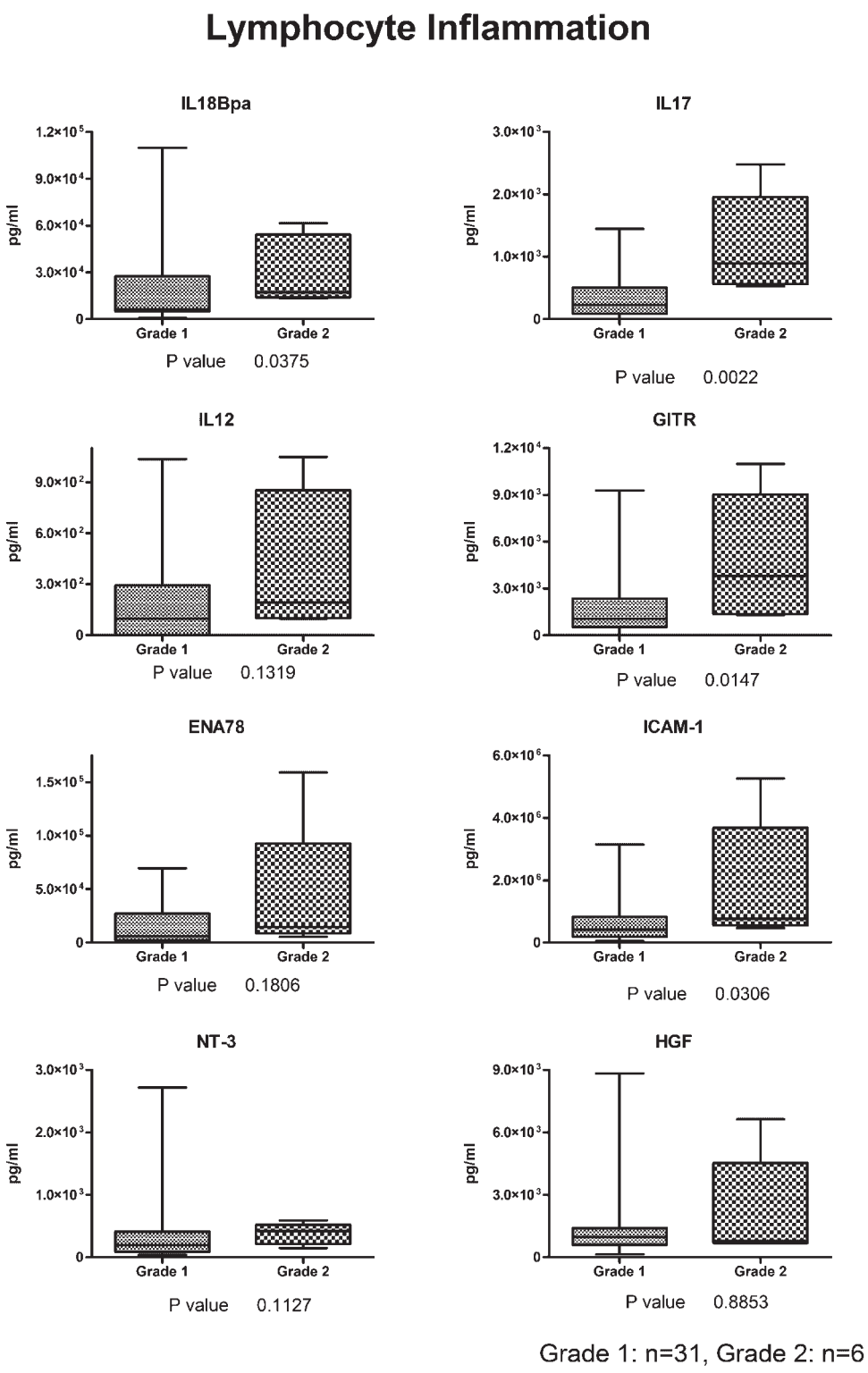

Fig. 5. Relationship between cytokine levels and lymphocyte inflammation. ILI8Bpa, ILI7, GITR, and ICAM-I were significantly elevated in grade 2 lymphocytic inflammation ($P < 0.05$).

interferon gamma, and IL18Bpa limits the inflammatory response induced by IL18 [27]. IL18Bpa is also secreted by colon cancer cell lines after interferon gamma stimulation [28], which suggests that prostate cancer cells themselves may also secrete IL18Bpa upon stimulation by lymphocyte-derived cytokines with the background of inflammation. Since IL18Bpa inhibits the antitumor cytokine IL-18, the finding of IL18Bpa in

malignant prostates suggests an attempt by the cancer to escape immune surveillance and may be correlated with poor prognosis.

GITR has been shown to co-stimulate T cells and abrogate suppression of Treg [29], and to diminish NK cell antitumor immunity [30]. GITR also correlates with neutrophilic infiltration. In GITR−/− mice, neutrophil infiltration into arthritic areas was significantly less than in GITR+/+ mice [31]. Whereas GITR-expressing Treg help limit collateral tissue damage caused by vigorous antimicrobial immune response in normal tissues [32], Treg cells are increased in human solid tumors and an increased number of Treg cells correlates with poor prognosis [33]. The increase of GITR induced by the inflammation may be associated with the increase of Treg which suppress the antitumor immunity.

We found strong correlations between the expression levels of certain cytokines: ICAM-1 and GITR, ICAM-1 and ENA78, and GITR and NT-3. ENA-78 strongly attracts neutrophils, and the adhesion of neutrophils to vessel walls or epithelial cells in an area of inflammation occurs via ICAM-1 [34]. It is plausible that GITR-expressing cells, such as regulatory T cells or NK cells, stimulate the expression of NT-3 or ICAM-1 on prostate cancer cells; it may also be that GITR-expressing cells also express ICAM-1 and/or NT-3. Elucidating the reasons for the correlation between these cytokine pairs will require further studies.

A limitation of our study is that we did not assess the cytokine profile of prostatic fluid derived from prostates that were completely benign. The reason for this is that radical prostatectomy (complete removal of the prostate) is not performed on patients without prostate cancer. One consideration was to analyze the cytokine profile of prostatic fluid derived from radical cystoprostatectomy cases in men shown pathologically not to have prostate cancer. However, these men by definition have high grade and/or muscle-invasive bladder cancer neighboring the prostate, which might result in a cytokine profile difficult to discriminate from that associated with urothelial cancer (which can also reside in the prostatic urethra). Rather than selecting such patients, we chose to make our comparisons between cases with very minimal prostate cancer (M) and those with prostate cancers of significant volume (E). Interestingly, one case in the M group was diagnosed with prostate cancer by biopsy, but had no cancer found in the radical prostatectomy specimen despite intensive re-sectioning. While this case cannot be considered a completely negative control for prostate cancer, analysis of prostatic fluids from this case by ELISA did not demonstrate any significant differences in comparison with the average data derived from the other M group cases. In addition, when we controlled for specimen weight as a surrogate of BPH, we did not note any significant differences in cytokine levels across all cases. An advantage to using RRP specimens for the source of prostatic fluids analyzed in this study is that the entire prostate has been carefully examined for cancer and inflammation in a way that is uniquely afforded by having the entire gland available through radical surgery. Although all of the minimal disease cases analyzed (one with exception) have cancer, we are sure that it is a small amount (<15 mm^2). Thus, we feel that the cytokine profiles we have described delineate important differences between early, small cancers and late, more extensive cancers and regarding the related inflammatory status of the prostate.

CONCLUSIONS

We hope that our cytokine data provide information that is helpful to researchers studying cytokine networks, paracrine stimulation pathways, and oncogenesis in the prostate. HGF and IL18Bpa were elevated in prostatic fluid from patients with extensive prostate cancers. IL17, GITR, ICAM-1, and IL-18Bpa were elevated in prostatic fluid from specimens with neutrophil inflammation in gland lumina, and IL18Bpa, IL17, GITR, ICAM-1 were elevated in fluid from specimens with lymphocytic inflammation in stroma. These and other cytokines may perhaps be useful in early detection and prognostication efforts if they are found not only in prostatic fluid obtained ex vivo, but in expressed prostatic secretions or post-DRE urine samples from patients with their prostates till in situ.

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Endoglin (CD105) as a Urinary and Serum Marker of Prostate Cancer

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Abstract

Purpose: To evaluate endoglin (CD105) as a prostate cancer biomarker using urine and serum samples collected from men with and without prostate cancer.

Experimental Design: 99 men with indications for prostate biopsy provided urine samples after DRE. Serum samples were collected from 20 men without prostate cancer and at low risk for the disease, and from 69 men with prostate cancer who subsequently underwent radical prostatectomy (30 pT2, 39 pT3). Endoglin levels were assessed by ELISA.

Results: Urinary endoglin was elevated in men with biopsy-positive prostate cancer compared to biopsy-negative men (p=0.0014). Urinary endoglin levels in men with prostate cancer correlated with primary tumor volume at radical prostatectomy. The area under the receiver-operator characteristics (ROC) curve was 0.72 for urinary endoglin and 0.50 for serum PSA. Sensitivity for cancer detection was 73% and specificity was 63%. There were no differences in serum endoglin between normal and cancer cases, but there were increases in serum endoglin in non-organ confined (NOC, pT3) vs. organ-confined (OC, pT2) cases (p=0.0004). The area under the ROC curve was 0.75 for serum endoglin and 0.63 for PSA for predicting NOC status, with a sensitivity of 67% and a specificity of 80% at a serum endoglin cutoff of 17 ng/ml.

Conclusions: Elevations in post-DRE urinary endoglin levels suggest that there may be value in further studying endoglin as a urinary biomarker of prostate cancer. Endoglin levels in both urine and serum may aid in the noninvasive detection and prognostication of prostate cancer.

Introduction

Prostate cancer is known to be clinically heterogeneous, with some cases presenting in an indolent fashion and others widely metastatic at diagnosis. PSA, DRE and biopsy Gleason score are the three clinical tools typically used to stratify newly diagnosed men into low, intermediate, or high-risk prognostic groups.[1] No other marker in routine use significantly adds to either the diagnostic or prognostic power of these clinical parameters. Nevertheless, there is a need for additional markers of early or aggressive/advanced prostate cancer, and the search for these is ongoing and increasingly technology-driven.[2,3, 4]

We have previously used a human cytokine array to identify cytokines in expressed prostatic fluid associated with large volume prostate cancers. We found that a variety of growth factors, cytokines, and markers of angiogenesis were up-regulated in prostatic fluid from such cases.[5] One of the 20 most-upregulated molecules (see ref. 5

Appendix) was endoglin (CD105), a type I homodimeric integral transmembrane glycoprotein and accessory TGF-β receptor; another was the endoglin ligand activin-A.[6] Given these common pathway findings, we selected endoglin for further study.

Endoglin is primarily expressed in proliferating vascular endothelial and smooth muscle cells, and is highly expressed on endothelial cells during tumor angiogenesis and inflammation. It has weak or negative expression in normal tissues. Endoglin is expressed in prostate microvasculature in association with prostate cancer, and is increased in the serum of patients with colorectal, breast and lung cancer metastases.[7,8] Immunohistochemical analysis has shown endoglin to be expressed not only by endothelium associated with prostate cancer, but also by some PIN and prostate cancer epithelial cells and associated stromal components.[9] Recently, soluble endoglin has been shown to be of independent prognostic value as a serum indicator of prostate cancer metastasis to pelvic lymph nodes and of biochemical recurrence after prostatectomy.[10,11] Whether endoglin may serve as a marker for prostate cancer in locally-derived tissue (biopsies), or biofluids (expressed prostatic secretions, post-DRE urine) has been little studied.

We set out to assess whether endoglin levels could predict the presence of prostate cancer and/or correlate with advanced disease. Since endoglin is a local marker

of vascular proliferation in response to injury and/or angiogenic stimulation, we felt that assessing endoglin levels from the prostatic microenvironment more directly might have merit: To this effect we assayed urine samples collected following digital rectal examination (DRE) which is known to be enriched with prostatic secretions, from patients with and without prostate cancer. In addition, we assessed endoglin in archival serum samples from men with and without prostate cancer in order to assess its potential as a cancer biomarker.

Materials and Methods

Sample collection

Urine samples were collected in the Urology Clinic. Approval was obtained from our Institutional Review Board before initiating the study and all patients provided written informed consent prior to providing urine samples. Initial voided urine specimens (10 to 100ml) were prospectively collected from 99 men with an indication for prostate biopsy immediately following DRE during a single office visit. Voided urine specimens were kept at 4^{o}C for up to 4 hours prior to centrifugation for 10min at 1000g to remove sediments and then urine supernatants were kept at -80^{o}C until analysis. In addition, 89 archival serum samples were obtained from our biorepository and linked to information about patient prostate health status and other relevant

demographic and pathologic data.

Enzyme-Linked Immunosorbent Assay

Endoglin levels were measured by enzyme-linked immunosorbent assay (ELISA). A human Duo set (R&D Systems, Minneapolis, MN)) was used to detect endoglin in urine and serum. Briefly, 96-well microplates were coated with capture antibody and incubated overnight. After the blocking with 10%BSA in PBS for urine and 25% FBS in PBS for serum, samples were added (100µl/well) in duplicate for incubation for 2 hrs at room temperature. Detection antibodies were subsequently added (100µl/well)) and incubated for 2 hrs at room temperature. Incubation with streptavidin-horseradish-peroxidase (for 20 min) was followed by detection with 3,3V,5,5V-tetramethylbenzidine (TMB) for 20 min. The reaction was stopped by the addition of 1.5 M H_2SO_4. Plates were read at 450 nm wavelength on a microplate reader (PHERA star, BMG Labtech, Durham, NC). All reactions were done at room temperature. Serum samples were assayed at a 4-fold dilution.

ELISA data from urine samples were normalized by total urinary protein or urinary creatinine levels as measured by Dade Dimension RxL. Serum ELISA data were not normalized.

These data were analyzed by cancer grade on biopsy (Gleason score 6 vs. $\geq$7)

and, for the 36 radical prostatectomy cases, by pathologic stage, pathologic grade (Gleason score 6 vs. $\geq$7), and tumor volume (minimal-moderate vs. extensive). Specimens with minute foci of cancer or a maximum tumor area < 15 mm^2 were termed "minimal" disease, while specimens with a maximum tumor area > 80mm^2 were termed "extensive" disease; tumors of in-between sizes were termed "moderate" disease.[5]

Data Analysis and Statistics

Statistical analyses were done using GraphPad Prizm 4.0 for Windows. Mann-Whitney tests were used to analyze the difference of 2 categories. Power calculations were performed based on available serum endoglin levels in the literature. [8, 10, 11] With a limited number of patients in the control group (20), having 65 patients in the prostate cancer group and a two-sided alpha = 0.05 resulted in power >0.90 to detect a statistical difference. Biochemical and clinical prostate cancer recurrence data were available for 39 patients with non-organ confined disease. Kaplan-Meier recurrence curves were generated for cases with low (< median) and high (> median) serum endoglin levels, and Log-Rank tests were used to analyze the differences. Statistical significance was defined as a p value < 0.05.

Results

Endoglin Levels in Urine

ELISA was used to quantitate the levels of post-DRE urinary endoglin in a 99-man cohort of men at increased risk of prostate cancer. Of these 99 men, 67 had a biopsy positive for prostate cancer, and 32 were biopsy-negative. The men with and without biopsy-positive prostate cancer were well-matched by age, PSA and DRE findings (Table 1A).

Endoglin levels were significantly higher in the urine of men with prostate cancer than in those without prostate cancer (Figure 1A). Endoglin levels were normalized both to total urinary protein (TP) (Figure 1B) and to urinary creatinine (Figure 1C), but remained significantly elevated in the cancer cases regardless of the method of normalization (though normalization to total urinary protein was most discriminating). In order to assess whether endoglin levels might confer prognostic information in patients diagnosed with prostate cancer, we stratified those who underwent radical prostatectomy (n=34) by stage (organ-confined (OC), non-organ confined (NOC)), Gleason score ($\leq$6, $\geq$7), and tumor volume (minimal-moderate, extensive). Urinary endoglin levels were significantly higher in cases with high tumor volume (extensive prostate cancer, mean endoglin level = 9.73pg/μg ± 7.35, range 0 – 25.95) compared to cases with smaller tumor volume (minimal/moderate prostate cancer, mean endoglin level = 3.25 pg/μg ± 5.05, range 0 – 23.4) p=0.008 (Figure 2).

Mean urinary endoglin in men without prostate cancer was 73.2pg/ml ± 77.0 (range 0 – 274.8), and in those with prostate cancer was 132.4pg/ml ± 121.4 (range 0 – 608.3) (p = 0.0135). Mean endoglin levels normalized by TP of men without prostate cancer were 5.18 pg/μg ± 6.8 (range 0 – 27.7), and those with prostate cancer were 13.4 pg/μg ± 14.4 (range 0 – 86.7) (p = 0.0006). Mean endoglin levels normalized by urinary creatinine of men without prostate cancer were 0.92 pg/ml*dl/mg ± 1.17 (range 0 – 4.02), and those with prostate cancer were 1.75 pg/ml* dl/mg ± 1.76 (range 0 – 7.78) (p = 0.0077). There were no significant differences in urinary endoglin levels by Gleason score or cancer stage (data not shown). Urinary endoglin levels did not correlate with serum PSA or age.

The area under the receiver-operator characteristics (ROC) curve (AUC) for urinary endoglin was 0.72 (95% CI 0.61 – 0.82), in contrast to an AUC for PSA of 0.50 (95% CI 0.37 – 0.63) (AUC comparison $p<0.01$) for cancer detection in our patient cohort (Figure 3). The sensitivity and specificity at different endoglin/urinary TP cutoffs are listed on Table 2.

Endoglin Levels in Serum

Serum samples in a separate cohort of 89 patients with and without prostate cancer were also assessed for endoglin levels by ELISA (Table 1B). There was no

overall difference in serum endoglin levels in men with prostate cancer compared to men without prostate cancer (16.9ng/ml ± 2.6, range 9.4 – 25.5 vs. 18.1ng/ml ± 2.6, range 13.8 – 21.6, respectively) (Figure 4). However, among the 69 men with prostate cancer, endoglin levels were significantly higher in NOC (mean 18.0 ng/ml ± 3.6, range 9.4 – 25.5) vs. OC disease (mean 15.4ng/ml ± 2.3, range 11.5 – 20.2) ($p<0.01$). The men with prostate cancer were typically older, had higher PSA, and had more abnormal DRE findings than the men who did not have prostate cancer (Table 1B), but in separate univariate analyses, no correlation was found between serum endoglin levels and age or Gleason score. The ROC curve for serum endoglin is compared to that for PSA to predict NOC disease (Figure 5A), with an AUC for endoglin of 0.75 (95% CI 0.63 – 0.87) in contrast to an AUC for PSA of 0.63 (95% CI 0.50 – 0.77) (AUC comparison $p=0.10$). The sensitivity was 67% and the specificity was 80% for the prediction of non-organ-confined disease with a serum endoglin cutoff of 17.0ng/ml.

A subset of patients with NOC disease with (20) and without (19) postoperative PSA recurrence was compared by preoperative serum endoglin level, and no difference was found (18.6 vs. 17.3 ng/mL). Log Rank analysis for post-prostatectomy biochemical recurrence showed no significant difference between men in this subset with low versus high endoglin levels (<50%ile vs. >50%ile endoglin,

p = 0.21) (Figure 5B).

Discussion

We hypothesize that biomarkers associated with the development of prostate cancer and/or of its dedifferentiation can be measured from the prostatic microenvironment. Prostatic stroma and epithelium are known to be rich sources of cytokines and growth factors involved in the regulation of prostatic development, hypertrophy, and neoplasia, as well as of inflammation and local immunity.[12] In previous experiments, we assayed prostatic fluid for cancer-associated proteins: In addition to increased amounts of cytokines such as HGF and IL18 binding protein-a, we noted increased CD105/endoglin and increased amounts of one of its ligands (activin-A) in expressed prostatic fluid collected from radical prostatectomy specimens with large volume cancers.[5] In the present study, we show that endoglin is increased in urine collected after DRE from men with prostate cancer on biopsy compared to men without prostate cancer, and that post-DRE urinary endoglin levels are predictive of prostate cancer in a cohort of men at increased risk by PSA and DRE criteria (Figure 3). This is the first assessment of the ability of endoglin to distinguish between benign and malignant prostate disease. In addition, endoglin levels measured from serum were

predictive of non-organ confined prostate cancer using an archival set of serum samples from men with and without prostate cancer.

Endoglin was assayed in the urine after DRE in order to directly (but minimally-invasively) assess its presence in the prostatic microenvironment *in vivo*. A DRE exerts pressure on much of the prostate, and at least in theory allows for a sampling of secretions from the entire gland, unlike a prostate biopsy. It is known that initial voided urine obtained after DRE is enriched in prostatic proteins.[13] We did not specifically assess whether the urinary endoglin we detected was a result of circulating and filtered endoglin or a result of local prostatic endoglin. However, given that the assays were performed after prostatic manipulation, that we have previously found endoglin in expressed prostatic secretions, that only initial urine was collected as it coursed through the prostate after prostatic examination ("Voided bladder 3" samples, per Meares-Stamey),[13] and that we normalized to total protein in the urine samples (which mostly comes from prostatic sources after a DRE), we surmise that the endoglin we assayed was predominantly of prostatic origin. Urine is likely to become an increasingly powerful source of prostate-specific biomarkers,[2,4] but until quantitative detection methods improve it may be reasonable to sample urine enriched in prostatic secretions rather than urine that is prostate secretion-poor (such as mid-stream

urinalysis).

Serum PSA is an extremely powerful marker of prostatic disease, with tremendous diagnostic and prognostic utility, but it is not cancer-specific.[14] Nevertheless, PSA and its isoforms are the sole prostatic serum markers in clinical use today, and PSA testing alone has changed the epidemiology of prostate cancer dramatically since its introduction in the 1980s.[15] Our cohort of men who were biopsied and who provided post-DRE urine samples had mean PSA levels between 5 and 5.5 ng/ml (i.e. elevated), and almost 20% had abnormal DRE findings (Table 1). These men could be characterized as being at elevated risk for prostate cancer primarily based on PSA criteria. Our urinary endoglin test demonstrated better performance characteristics than PSA in this cohort of high-risk men; however, it is unclear how urinary endoglin would perform in a patient population at normal risk for prostate cancer, where PSA retains significant clinical utility.

We also studied endoglin levels from archival serum samples in a separate cohort of men with and without prostate cancer. Serum endoglin levels in pathologic stage III (NOC) disease were significantly greater than those in pathologic stage II (OC) disease, though the absolute levels did not differ greatly. This statistically significant finding may not easily translate into a clinically useful pretreatment counseling tool

because of the small differences in absolute levels and also because comparably high serum endoglin levels were noted in both NOC cases and in men without prostate cancer.

Endoglin has recently gained attention in prostate cancer prognostication by work from the group from the University of Texas Southwestern that has had a longstanding interest in TGF-β related proteins and prostate cancer. They analyzed endoglin levels in archival serum from a large cohort of prostatectomy patients and showed an independent association between increased plasma endoglin and the presence of lymph node metastasis and biochemical recurrence after prostatectomy, suggesting this molecule may be a marker of and/or facilitate extraprostatic spread.[10,11] Interestingly, our two groups have come upon endoglin in distinct manners, one from scientific analysis of TGF-β related pathways, and the other from cytokine profiling of prostatic fluid; consistently, both have demonstrated associations between endoglin and aggressive prostate cancer.

Since endoglin is a marker of pan-endothelial damage and angiogenesis, it is unlikely that circulating endoglin levels would be significantly affected by localized prostatic disease states - indeed, serum endoglin levels are affected by cardiovascular disease status, cholesteremia, and cirrhosis.[16,17] However, circulating endoglin is

increased in metastatic disease states.[8,10, 11] Presumably, the angiogenic cascade necessary for metastasis is associated with systemic dysregulation of the TGF-β superfamily that results in an increase in detectable serum endoglin. Our finding of increased serum endoglin in non-organ confined prostate cancer states is consistent with the notion of endoglin as a marker of advanced disease and supports the dramatic associations found between endoglin and metastatic disease by the U.T. Southwestern group. However, we were unable to show increased endoglin levels in patients with prostate cancer compared to patients to without it. In addition, serum endoglin levels in our study patients differed from those in the other studies, which were somewhat higher even in localized disease states (20-40ng/ml).[10, 11] Levels in our cohort ranged between 7.5 and 27.5 ng/ml (Figure 4), while in the cardiovascular literature, levels in normal controls and in patients with familial atherosclerosis and/or in the setting of myocardial infarction average between 3 and 8 ng/ml.[16, 17] There is no standard assay for endoglin, but a variety of kits and antibodies are commercially available; it is possible that the specific ELISA used may be responsible for the range of levels reported in these different studies. Alternate explanations are that endoglin levels in serum and plasma may differ, and that endoglin levels may be affected by time of archival storage.

Endoglin's molecular role if any in prostate carcinogenesis and metastasis is

unknown. Endoglin is known to be strongly up-regulated in the endothelium of various tumors compared with normal tissues, suggesting that endoglin plays a significant role in tumor angiogenesis.[18] Hypoxia transcriptionally induces endoglin expression via HIF-1, expression which is enhanced in the hypoxic setting by TGF-β.[19] In turn, endoglin antagonizes the inhibitory effects of TGF-β1 on human vascular endothelial cells; indeed normal cellular levels of endoglin/CD105 are required for the formation of new blood vessels.[20] Future work is required to determine the specific source of the endoglin detectable in the urine of prostate cancer patients, if it is bioactive, and what are its most important downstream targets with respect to prostate oncogenesis and prostate cancer progression.

Conclusions

Endoglin is an accessory TGF-β receptor transmembrane glycoprotein associated with angiogenesis and prostatic neoplasia that is present in prostatic fluid. Urinary levels of endoglin are increased in men with prostate cancer compared to levels in men without prostate cancer, and serum endoglin levels may correlate with increasing prostate cancer stage. Further studies are necessary to validate these initial observations.

Tables

Table 1. Patient Characteristics Respective to Analyzed Urine and Serum Samples

A.		Urine samples		
		Negative biopsy	Positive biopsy	
No. pts		32	67	
Median age (range)		62 (40-81)	60 (45-84)	p=0.31
Median PSA (ng/ml) (range)		5.4 (0.6-11.5)	5.05 (1.7-20.5)	p=0.98
Suspicious DRE (%)		18.7	19.4	
Gleason Score	6	-	43 (64%)	
	7	-	21 (31%)	
	8	-	1 (2%)	
	9	-	2 (3%)	

B.		Serum samples				
		Controls	CaP Patients			
			All		OC	NOC
No. pts		20	69		30	39
Median age (range)		56 (46-66)	61 (47-69)	p=0.10	57.5 (48-66)	62 (47-69)
Median PSA (ng/ml) (range)		1.05 (0.3-1.9)	5.29 (0.9-27.8)	p<0.01	4.7 (2.9-13.4)	6.5 (0.9-27.8)
Suspicious DRE (%)		0	24.3		13.3	32.5
Gleason Score	6	-	27 (39%)		17	10
	7	-	35 (51%)		13	22
	8	-	5 (7%)		0	5
	9	-	2 (3%)		0	2

Table 2. Urinary endoglin normalized to total urinary protein (TP) as a marker for prostate cancer in men at increased risk for prostate cancer (abnormal PSA &/or DRE)

Urinary Endoglin/TP Cutoff	Sensitivity		Specificity	
	%	95% CI	%	95% CI
14.8	34.3	(23.1 - 46.9)	93.7	(79.1 - 99.2)
8.9	53.7	(41.1 - 66.0)	84.3	(67.2 - 94.7)
4.0	73.1	(60.9 - 83.2)	62.5	(43.6 - 78.9)
3.1	80.6	(69.1 - 89.2)	50.0	(31.8 - 68.1)
1.9	85.0	(74.2 - 92.6)	43.8	(26.3 - 62.3)

Figure Legends

1. Urinary endoglin collected after DRE in patients who had either a negative (n=32) or positive (n=67) biopsy for prostate cancer. A) Urinary endoglin B) Urinary endoglin/Urinary total protein (TP), C) Urinary endoglin/Urinary creatinine (Cr).

2. Urinary endoglin/Urinary total protein in patients with prostate cancer who subsequently underwent radical prostatectomy and had tumor volume estimated.

3. Receiver operating characteristic curves of urinary endoglin and serum PSA for the detection of cancer in our cohort.

4. Serum endoglin levels in patients A) without prostate cancer (Normal, n=20) and with prostate cancer (Cancer, n=69), and B) with organ-confined prostate cancer (OC, n=30), and with non-organ confined prostate cancer (NOC, n=39).

5. A) ROC curve of serum endoglin and serum PSA for the prediction of non-organ confined disease in patients with prostate cancer on biopsy. B) Kaplan-Meier recurrence curves for cases with low (< median) and high (> median) serum endoglin levels for 39 cases with documented non-organ confined disease.

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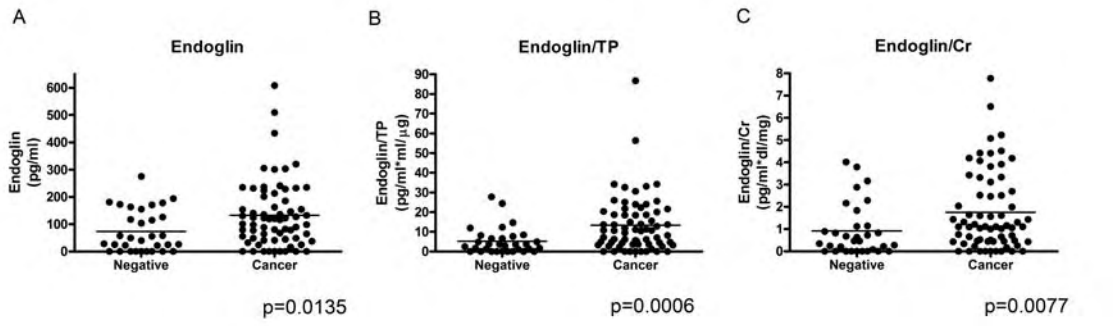

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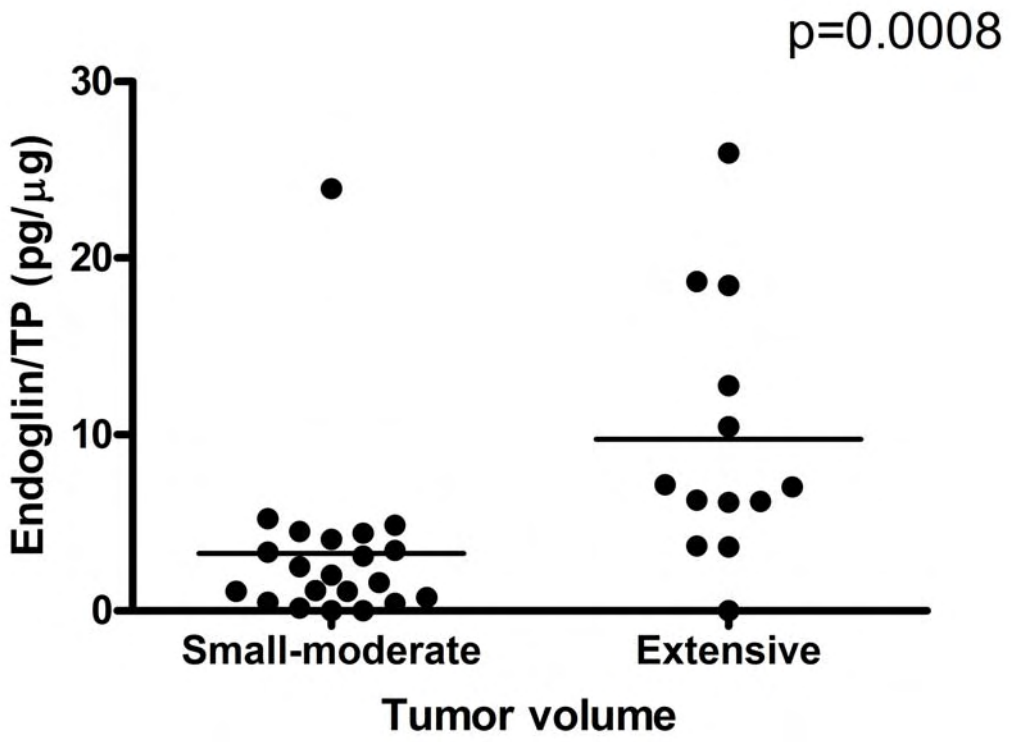

Figure 2. Urinary endoglin/Urinary total protein in patients with prostate cancer who subsequently underwent radical prostatectomy and had tumor volume estimated.

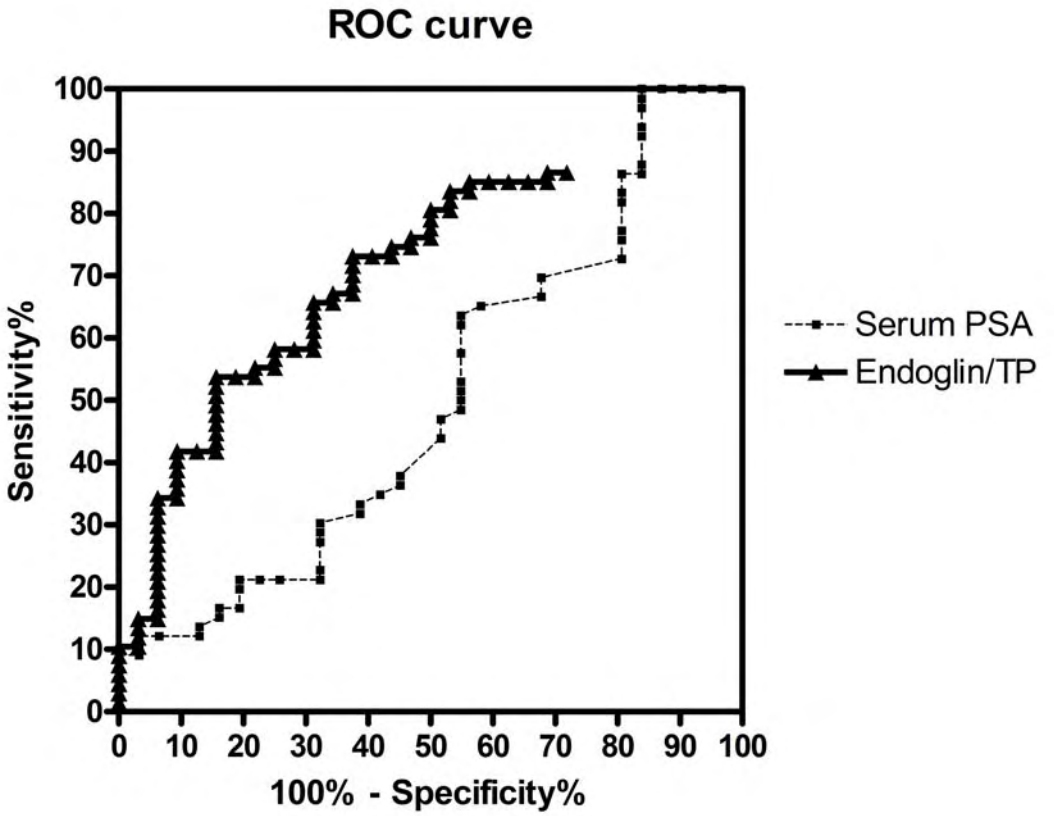

Figure 3. Receiver operating characteristic curves of urinary endoglin and serum PSA for the detection of cancer in our cohort.

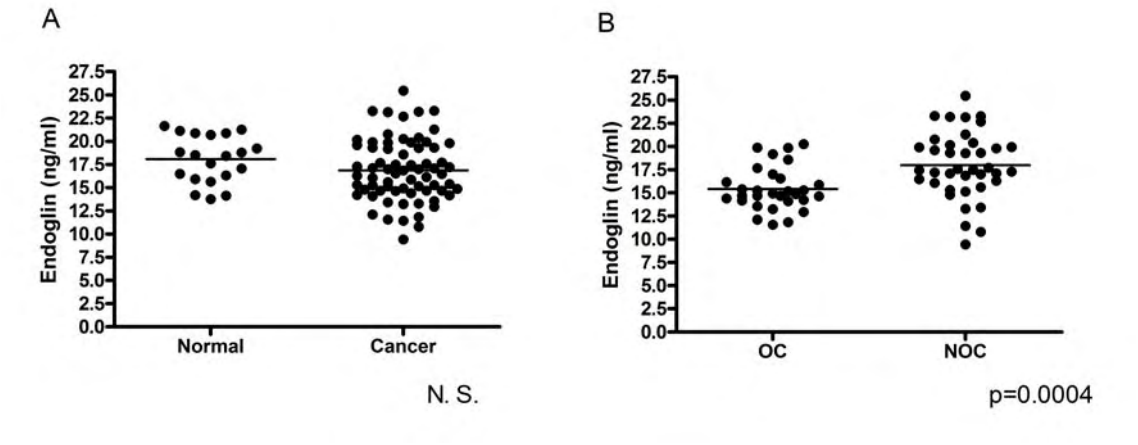

Figure 4. Serum endoglin levels in patients A) without prostate cancer (Normal, n=20) and with prostate cancer (Cancer, n=69), and B) with organ-confined prostate cancer (OC, n=30), and with non-organ confined prostate cancer (NOC, n=39).

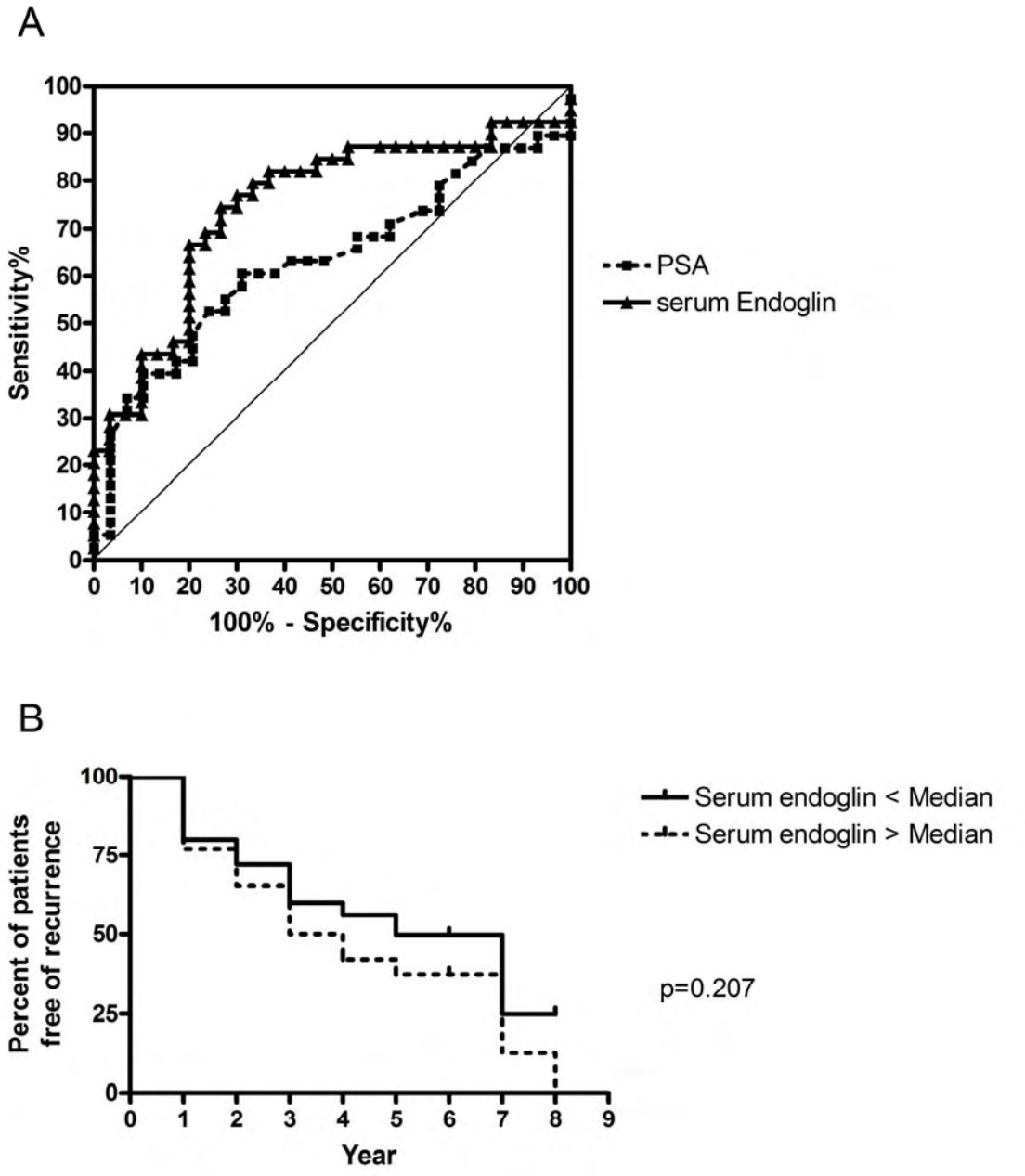

Figure 5. 5. A) ROC curve of serum endoglin and serum PSA for the prediction of non-organ confined disease in patients with prostate cancer on biopsy. B) Kaplan-Meier recurrence curves for cases with low (< median) and high (> median) serum endoglin

levels for 39 cases with documented non-organ confined disease.

IL18 binding protein is produced by prostate cancer cells and its levels in urine and serum correlate with tumor status.

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Abstract

Cytokines may play a role in the initiation and progression of prostate cancer. A cytokine antibody array was applied to prostatic fluid obtained from patients with prostate cancer, and interleukin 18 binding protein a (IL18Bpa), a potent inhibitor of interleukin 18 secreted mainly by monocytes, was noted to be significantly upregulated in cases with large volume disease. We sought to further characterize the association of IL18Bpa with prostate cancer and determine whether IL18BPa levels in patient serum and urine samples had clinical relevance. IL18Bpa was expressed and secreted by the prostate cancer cell lines DU145 and PC3, but not by LNCaP and CWR22, upon interferon-γ (IFN-γ) stimulation. IFN-γ-induced secretion of IL18Bpa was enhanced by added TNF-α, IFN- α and IFN-β. The IL18Bpa secreted from DU145 and PC3 was functionally inhibited IL18. Conditioned medium from IL18Bpa-overexpressed PC3 cells suppressed $CD8^+$ IFN-γ^+ cells and $T_H1$cells in human peripheral blood. Immunohistochemical analyses showed positive IL18Bpa staining in prostate cancer cells as well as in macrophages in radical prostatectomy specimens. Significant differences in post-DRE urinary IL18Bpa levels (normalized by total protein) were found between cases with and without cancer on biopsy ($p=0.02$) and serum IL18Bpa levels correlated with Gleason score ($p=0.03$). Our finding of elevated IL18Bpa secretion from prostate cancer cells suggests an attempt by cancer to escape immune surveillance. IL18Bpa merits further study as a marker of aggressive prostate cancer and as a therapeutic target.

Introduction

Chronic inflammation is commonly observed in radical prostatectomy specimens, and prostate tissues often contain increased inflammatory infiltrates, including T cells, B cells, macrophage, neutrophils and mast cells (1, 2). The immune system can specifically identify and eliminate tumor cells: Tumor infiltration by T cells, NK cells, and/or NKT cells is associated with an improved prognosis for a number of different tumor types (3, 4). However, the immune system has a paradoxical role in tumor development, as it has been established that chronic activation of innate immune cells, such as macrophages, mast cells and neutrophils, contributes to cancer development (4). Tumor cells may also create an immunosuppressive environment in cancer patients, and may escape immune surveillance through various mechanisms (5). Cytokines secreted by tumor and inflammatory/immune cells are one factor that can promote tumor development and tumor cell survival in an otherwise immunologically intact host (3).

In this study, we initially chose to use a human cytokine array to search for cytokines in prostatic fluid that may be associated with aggressive prostate cancer, and found that interleukin 18 binding protein a (IL18Bpa) was significantly upregulated in cases with large volume disease. IL18Bpa is a secreted glycoprotein possessing high-affinity binding and an ability to neutralize Interleukin-18 (IL18) (6). IL18 in turn is a mediator of T_H1 cytokines, induces high levels of interferon gamma (IFN-γ) secretion by both NK cells and T_H1 cells, potentiates IL-12-induced T_H1

development, and plays an important role in T-cell proliferation. It also enhances FasL-mediated cytotoxicity of NK cells and T_H1 cells, and has proinflammatory properties such as inducing macrophage chemotactic molecules. In mice, IL18 exerts its anti-tumor activity via IFN-γ, NK cells and $CD4^+$ Fas-dependent cytotoxicity. The IL18 binder IL18Bpa is thought to form part of a negative feedback loop designed to limit T_H1 immune activation. IL18Bpa is constitutively expressed in human spleen and leukocytes, with monocytes the primary source of IL18Bpa, and keratinocytes, renal mesangial cells, and colon cancer/epithelial cells also reportedly express the binding protein (7, 8). In this study, we sought to further characterize the association of IL18Bpa with prostate cancer and assess whether its presence in patient serum and urine samples had clinical relevance.

Materials and Methods

Cell culture. Cell lines. LNCaP, PC3, DU145, CWR22 and KG-1 were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37C containing 5% CO_2.

Sample collection. All samples were collected using IRB-approved protocols. Prostatic fluids were collected by squeezing *ex vivo* prostate glands freshly obtained following radical prostatectomy for prostate cancer. Prostate glands with either minimal prostate cancer (M, n=20) or

extensive prostate cancer (E, n=20) as estimated by pathologic tumor volume were chosen for this study. Specimens with minute foci of a maximum tumor area of less than 15mm^2 were assigned to the M group, and specimens with a maximum tumor area of more than 80mm^2 were assigned to the E group. Frozen prostate tissues were obtained from an institutional tumor bank from preserved radical prostatectomy specimens. The prostate cancer tissues assessed consisted of Gleason 6: n= 4, Gleason 7: n=1, Gleason 8: n=1, and Gleason 9: n=1 cancers. Six benign areas from the radical prostatectomy specimens were also obtained and assessed. Urine samples were collected in the Urology Clinic. Initial voided urine specimens (10 to 100ml) were prospectively collected from 99 men with an indication for prostate biopsy immediately following DRE during a single office visit. Voided urine specimens were kept at 4^{o}C for up to 4 hours prior to centrifugation for 10min at 1000g to remove sediments and then urine supernatants were kept at -80^{o}C until analysis. In addition, 89 archival serum samples were obtained from our biorepository and linked to information about patient prostate health status and other relevant demographic and pathologic data.

Cytokine Antibody Array. A Raybio^{TM} Human Cytokine Array kit (Raybiotech, Norcross, GA, USA) including 174 cytokines was used per the manufacturer's recommendations. Positive control signals on each membrane were used to normalize cytokine signal intensities from cytokine antibody arrays. Then, the data was normalized to PSA levels in each prostatic fluid sample to account for differential yields of fluid actually of prostatic origin. The normalized intensity value

of cytokines in each group (M or E) was converted into the relative n-fold change between groups.

Reverse-transcriptase PCR. Total RNA was extracted from LNCaP, CWR22, PC3 and DU145 after 24 hr incubation with or without 10ng/ml IFN-γ or from frozen prostate tissues with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (1 µg) was treated with DNase I (Invitrogen). First-strand cDNA was produced with random hexamers as per the manufacturer's recommendations (Omniscript RT kit/Qiagen). PCR amplification of *IL18Bpa, GAPDH, JAK1 and JAK2* was done with HotStar Taq Plus Master Mix (Qiagen). Primers used were as follows: *IL18Bpa*, 5'-ATGGAACGCTGAGCTTATCCT-3' (forward) and 5'-GGCCCTGTGCTGAGTCTTA-3' (reverse); *GAPDH*, 5'- ACCAGGGCTGCTTTTAACTCT -3' (forward) and 5'-GATGACAAGCTTCCCGTTCT -3' (reverse); *JAK1*, 5'-GGCGTCATTCTCCAAAGAAGC -3' (forward) and 5'-TCAACAGAAACAACATTTGGT -3' (reverse); *JAK2*, 5'-AACCTCACAAACATTACAGAG -3' (forward) and 5'-GATTTCCTGTCTTCCTGTCTT -3' (reverse). Amplification conditions were as follows: 15 minutes at 95°C (one cycle) and 45 seconds at 94°C; 45 seconds at the annealing temperature (60°C for *IL18Bpa* and *GAPDH*, 54°C for *JAK1 and* 50°C for *JAK2*); and 60 seconds at 72°C (32 cycles for IL18Bpa and GAPDH and 35 cycles for *JAK1* and *JAK2*) and 72°C for 5 minutes (one cycle).

Western blot analysis. After washing with ice-cold PBS, cells were harvested in RIPA buffer (Pierce, Rockford, IL) supplemented with Halt protease inhibitor cocktail (Pierce). Total

cellular protein concentrations were determined by using a BCA protein assay reagent (Pierce). 60μg protein of lysates were subjected to SDS–PAGE under the reducing condition, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with monoclonal anti-human IL18Bpa antibodies (R&D Systems, Minneapolis, MN) followed by horseradish peroxidase-conjugated secondary antibodies, and developed with the Super Signal West Dura Extended Duration Substrate kit (Pierce).

Measurement of IL18Bpa in cell culture conditioned media. $1x10^5$ of each cell type were seeded in 24-well plates and 24hr later media were changed to media containing 0, 0.4, 2.0, 10, 50ng/ml of IFN-γ (R&D Systems). After 24hrs of incubation, supernatants were collected, centrifuged at 1000g for 10min to remove cells and kept at -20°C freezer till analysis. $1x10^5$ of each cell type were also seeded in 24-well plates, and after incubation for 24hr at 37°C, media were changed to media containing 10ng/ml of IFN-γ. After 0, 12, 24, 48, and 72hrs of incubation, supernatants were collected. $2x10^4$ of each cells were seeded in 96-well plates (Falcon), and after incubation for 24hr at 37°C, media were changed to media containing 10ng/ml IFN-γ, 200ng/ml IL-12 (R&D Systems), 200ng/ml TNF-α (R&D Systems), 10000IU/ml IFN-α (R&D Systems) and 5000IU/ml IFN-β (R&D Systems) with or without 10ng/ml IFN-γ. After 24hrs of incubation, plates were centrifuged at 1000g for 10min, and supernatants were collected and subjected to ELISA.

Enzyme-Linked Immunosorbent Assay (ELISA). IL18Bpa in cell culture supernatants,

urine and sera, IFN-γ in cell culture supernatants, and IL18 in sera were measured by ELISA. A DuoSet ELISA development system (R&D Systems) was used to detect IL18Bpa and IFN-γ. A human IL18 ELISA kit (MBL, Nagoya, Japan) was used to detect IL18 in serum. Each cytokine was measured based on the manufacturer's recommendations. Urine and serum samples were assayed at a 10-fold and 4-fold dilution, respectively. ELISA data from urine samples were normalized by total urinary protein levels as measured by Dade Dimension RxL. Cell supernatant and serum ELISA data were not normalized.

IL18Bpa biological assay. LNCaP, CWR22, DU145 and PC-3 were seeded to 75cm^2 flasks and stimulated by 10ng/ml IFN-γ for 24hr. Then, cultures were washed 3 times with HBSS, and incubated with 10% RPMI/FBS for 2 days. 1.2x10^6 cells of KG-1 were incubated with 100ul of these conditioned media with 20ng/ml TNF-α and 0ng/ml or 40ng/ml IL18 at 37℃. RPMI/10%FBS without the incubation with cells was used as a control. After 24hr incubation, supernatants from KG-1 cell cultures were collected and subjected to IFN-γ ELISA.

Immunohistochemical analysis. 5μm paraffin-embedded radical prostatectomy sections were subjected to immunohistochemistry, performed with the Powervision+ IHC Detection System (Vision BioSystems, Norwell MA) according to the manufacturer’s recommendations. The sections were deparaffinized and rehydrated, and after the slides were steamed for 40 min in Target Retrieval Solution (DakoCytomation, Carpinteria, CA) for antigen retrieval, endogenous peroxidase activity

was blocked with 3% H_2O_2. Slides were incubated with the Mouse anti-human IL18Bp monoclonal antibody overnight at 4°C. Staining was visualized using 3,3'-Diamino-benzidine (DAB) (Sigma, Saint Louis, MO, USA, FAST 3,3'-Diamino-benzidine Tablets) and slides were counterstained with hematoxylin. Human Tonsil was used as the positive control.

Construction of human recombinant IL18Bpa. The ORF for His tagged- IL18Bpa was amplified from a cDNA clone (SC110117, Origene, Rockville, MD) with the following primer set, Forward 5'-CACCatgagacacaactggacacca-3' (CACC designates the required gateway sequence), Reverse 5'-ttaatgatgatgatgatgaccctgctgctgtggactgc-3'. Utilizing the Gateway system (Invitrogen), the PCR product was cloned into the pENTR/D-TOPO, and then put into the Destination vector behind a CMV promoter (pDEST12.2). Stable clones were obtained by transfecting PC3 cells with the pDEST12.2/IL18Bp-his vector or empty vector by the Fugene 6 reagent (Roche). Stable transfectants were selected in 500 μg/ml neomycin (Invitrogen) for 4–5 weeks, and single cell subclones overexpressing IL18Bpa were isolated and expanded for use in experiments. IL18Bp-PC3 cells and empty-PC3 cells were incubated with CTL medium (RPMI, 1% L-glutamate, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, 10% heat-inactivated FCS, and 3.47 μL/L 14.4 mol/L β-mercaptoethanol) for 3 days. Conditioned media were harvested, centrifuged at 1500g for 10min and supernatants were kept in -80°C.

Isolation of CD4+ T cells and CD8+ T cells. $CD4^+$ T cells and $CD8^+$ T cells were

positively isolated using the Dynal CD4 Positive Isolation Kit and Dynal CD8 Positive Isolation Kit (Invitrogen) from 3ml whole blood from a healthy volunteer according to the manufacturer's recommendations. The purity of $CD4^+$ T cells and $CD8^+$ T cells was confirmed by flow cytometry using surface staining with mAb against CD4 (eBioscience, clone RPA-T4, San Diego, CA) and CD8 (eBioscience, clone RPA-T8). $1x10^6$ cells of $CD4^+$ T cells and $CD8^+$ T cells were resuspended in conditioned CTL media from empty-PC3 or IL18Bp-PC3 with 0.05 µg/ml of phorbol 12-myristate 13-acetate (PMA) and 0.5 µg/ml of ionomycin and incubated for 3 days in the incubator. Then, cells were resuspended in CTL with 20 U/ml IL-2 (Roche, Nutley, NJ) for 3 days and subjected to intracellular staining and cell proliferation assay.

Intracellular antibody staining and flow cytometry. $CD4^+$ T cells and $CD8^+$ T cells were stimulated for 4 h at 37°C in CTL medium with 0.05 µg/ml of PMA, 0.5 µg/ml of ionomycin 1:1,000 GolgiStop (BD Biosciences, San Diego, CA) prior to staining. Intracellular staining with directly conjugated mAbs against FoxP3 (PerCP-Cy5.5, eBioscience, clone PCH101) and IL-4 (PE, eBioscience, clone MP4-25D2) for $CD4^+$ T cells, IFN-γ (PE; eBioscience, clone 4S.B3) and IL-17 (PerCP-Cy5.5; eBioscience, clone 64Dec17) for $CD4^+$ T cells or IFN-γ (PE) for $CD8^+$ T cells was done using the eBioscience Human Regulatory T-cell Staining Kit and the manufacturer's recommended protocol. Surface staining with a PE-Cy5.5 labeled mAb to CD56 (eBioscience, clone MEM188) for $CD8^+$ T cells was done prior to cell permeabilization. Flow cytometry was conducted

using Guava EasyCyte Plus (Guava Technologies, Hayward , CA), and data were analyzed using Win MDI software.

Cell Proliferation Assays. After 3 day-incubation in CTL media with IL-2, $CD4^+$ T cells and $CD8^+$ T cells were seeded in 96-well plates under CTL medium and the cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was added to each well, as specified by the supplier (Roche). After 1-hr of incubation, WST-1 absorbance at 450 nm was measured.

Data Analysis and Statistics. Results were expressed as mean ± SD. Statistical analyses were done using GraphPad Prizm 4.0 for Windows. Mann-Whitney tests and Student's *t*-tests were used to analyze the difference of 2 categories in clinical samples (prostatic fluids, urine and serum) and in vitro experiments, respectively. Statistical significance was defined as a P value < 0.05.

Results

IL18BPa levels correlate with Cancer Status and Gleason score. We initially used a human cytokine array to search for cytokines in prostatic fluid that may be associated with aggressive prostate cancer. In comparison to cytokines found in small volume tumors, IL18Bpa was one of the most upregulated cytokines in prostatic fluid from cases with large volume disease (Table 1). IL18Bpa levels in prostatic fluids were subsequently confirmed qualitatively by ELISA. IL18Bpa was elevated in cases with large volume prostate cancers (E group: extensive disease) compared to cases with

minimal cancer at radical prostatectomy (the M group: minimal disease) (P = 0.016) (Fig. 1*A*). In an analysis based on Gleason score, IL18Bpa was also significantly elevated in specimens with high Gleason grade (≥7) (P = 0.046) (Fig. 1*B*).

Prostate tissues and prostate cancer cell lines express IL18Bpa. RT-PCR analysis showed that IL18Bpa was expressed by prostate cancer from radical prostatectomy specimens and also from normal areas of prostates with prostate cancer (Fig. 2*A*). Expression analysis using the Oncomine Cancer Microarray database (http://www.oncomine.org) showed that IL18Bpa expression was significantly increased in lymph node metastases of prostate cancer (n=6) compared with non-metastatic prostate cancer cases (n=63) ($P < 0.01$) (Fig. 2*B*). Because IL18Bpa was strongly expressed by monocytes/macrophages (9) but is not known to be expressed by prostate cancer, we proceeded to analyze IL18Bp mRNA expression in prostate cancer cell lines by RT-PCR (Fig. 2*C*). PC3 strongly expressed IL18Bp mRNA, DU145 expressed it moderately, and LNCaP and CWR22 expressed it far less. Western blots of lysates of PC3 and DU145 revealed an approximately 42kDa band detected by monoclonal antibody against human IL18Bpa, consistent with the size of human IL18Bpa. This band was not detected in cell lysates from LNCaP and CWR22 (Fig. 2*D*).

PC3 and DU145 secrete IL18Bpa in response to IFN-γ stimulation. Since IL18Bp is a secretory protein, its levels in supernatants of LNCaP, CWR22, PC3 and DU145 were examined by ELISA. Since IFN-γ induces IL18Bp expression in monocytes and non-leukocytic cells, (7-9) we

stimulated prostate cancer cell lines with IFN-γ to examine IL18Bp secretion. 24hrs after IFN-γ stimulation, IL18Bp was detected in supernatants from PC3 and DU145 cell lines (but not in those from LNCaP and CWR22) in a dose- and time-dependent manner (Fig. 3*A&B*). Even after 72hrs of incubation, only low levels of IL18Bp were detected in the LNCaP supernatants. The effect of added androgen was also examined, but stimulation with 10nM R1881 and 10ng/ml IFN-γ did not induce IL18Bp secretion from the androgen-dependent cell lines LNCaP or CWR22 (data not shown). To confirm that the IL18Bp secreted from PC3 and DU145 was biologically active, conditioned media from LNCaP, CWR22, PC3 and DU145 were used to resuspend KG-1 cells (which secrete IFN-γ secondary to stimulation by IL18 and TNF-α) in 0ng/ml or 40ng/ml IL18 and 20ng/ml TNF-α. KG-1 cells incubated with conditioned media from LNCaP and CWR22 or control media together with IL18 and TNF-α secreted IFN-γ, but the secretion of IFN-γ from KG-1 cells was suppressed by the conditioned media from PC3 and DU145 (Fig. 3*C*). These data suggest that IFN-γ-stimulated PC3 and DU145 secrete biologically active IL18Bpa that suppresses IL18-induced production of IFN-γ from KG-1 cells.

In order to further study the reason some prostate cancer cell lines did and some did not secrete IL18BPa to IFN-γ stimulation, the expression of IL18Bpa mRNA was assessed 24hrs after IFN-γ stimulation. IL18BPa mRNA was highly upregulated in both PC3 and DU145, but barely upregulated in LNCaP and CWR22. The poor response of LNCaP and CWR22 to IFN-γ stimulation at the

messenger level may be the reason these lines do not secrete significant amounts of IL18Bpa.(Fig. 3*D*) Sodium butyrate (an inhibitor of both Ca2+ release from intracellular stores and of histone deacetylase (HDAC)) has been shown to suppress IFN-γ-induced IL18Bp expression in colon cancer cell lines (7). The HDAC-inhibitor Trichostatin A, as well as sodium butyrate also suppressed the IFN-γ-induced IL18Bp expression in PC3 and DU145 (data not shown). IFN-γ is known to exert its effect mainly through the Janus kinases JAK1/JAK2 which lead to STAT1 activation, and HDAC inhibitors prevent STAT1 activation through inhibition of JAK1 phosphorylation (10). The expression of JAK1 and JAK2 was examined in these prostate cancer cell lines by RT-PCR: LNCaP and CWR22 expressed very little JAK1 compared to PC3 and DU145, whereas JAK2 expression did not differ between cell lines. Low JAK1 expression is a possible explanation for the inability of LNCaP and CWR22 to secrete IL18BPa (Fig. 3*D*).

Secretion of IL18Bpa by IFN-γ stimulation is enhanced by TNF-α, IFN-α and IFN-β.

IL18Bp is induced from human peripheral mononuclear cells not only by IFN-γ but also by IL12 (9). We examined the effect of this other cytokine, IL12, as well as of TNF-α, IFN-α and IFN-β on secretion of IL18Bp from prostate cancer cell lines. No cytokine other than IFN-γ induced IL18Bp secretion from PC3 and DU145, but TNF-α, IFN-α, IFN-β enhanced IFN-γ-induced secretion of IL18Bp from DU145, and TNF-α enhanced it's secretion from PC3 ($P < 0.01$) (Fig. 4). No combination of these cytokines stimulated release of IL18Bp from LNCaP or CWR22 cells (data not

shown).

IL18Bp expression in prostate tissues. To determine the sources of IL18Bp expression in the prostate, we analyzed prostate specimens from patients with prostate cancer immunohistochemically. Tonsil was used as a positive control, where macrophages there are stained by anti-human IL18Bp monoclonal antibody (Fig. 5*A*). In prostate specimens, prostate cancer cells were positive and benign prostatic epithelial cells were negative, but in some of the glands with inflammation positively stained macrophages were found inside and around the glands (Fig. 5*B-D*). Thus, both prostate cancer cells and macrophages appear to be the main sources of IL18Bp in the cancerous prostate.

IL18Bp suppresses $CD8^+$ IFN-γ^+ cells and $T_H1$cells. As IL18 is a mediator of the T_H1 immune response, prostate cancer may secrete IL18Bp in order to escape immune surveillance. To examine the interaction of immune cells and prostate cancer via IL18Bp, transfected PC3 cells that secrete IL18Bp constitutively without IFN-γ were constructed. ELISA showed that the supernatant of these clones incubated for 48hrs without IFN-γ contained 4.97 ± 0.58 ng/ml IL18Bpa. $CD4^+$ cells and $CD8^+$ cells were isolated from the peripheral blood of a healthy volunteer at > 95% purity. $CD4^+$ or $CD8^+$ cells were incubated with conditioned media from IL18Bp-transfected PC3 or empty-vector-transfected PC3, and analyzed by flow cytometry (Fig. 6*A*,*B*). Conditioned media from IL18Bp-transfected PC3 suppressed $T_H1$cells ($CD4^+$IFN-γ^+) ($P < 0.05$) but there was no significant

change in T_H2 cells ($CD4^+IL\text{-}4^+$), T_H17 cells ($CD4^+IL\text{-}17^+$) or T_{reg} cells ($CD4^+FoxP3^+$) (Fig. 6*A*). Conditioned media from IL18Bp-transfected PC3 suppressed both IFN-γ^+CD56^- and IFN-γ^+CD56^+ cells ($P < 0.05$ and $P < 0.001$, respectively) (Fig. 6*B*). The proliferation of $CD8^+$ cells or $CD4^+$ cells was also measured after incubation with conditioned media followed by incubation with 10U/ml IL-2. Conditioned media from IL18Bp-transfected PC3 suppressed the proliferation of $CD4^+$ cells or $CD8^+$ cells compared to conditioned media from empty-PC3 ($P < 0.001$ and $P < 0.05$, respectively). Taken together, these results suggest that IL18Bp secreted from prostate cancer could suppress cytotoxic T lymphocytes (IFN-γ^+CD56^-), NK cells (IFN-γ^+CD56^+) and $T_H1$cells in vivo.

IL18Bpa levels in urine after DRE and serum. We analyzed IL18Bp levels both in the urine after prostatic manipulation (DRE) and in serum, in order to examine any correlations between IL18Bp and prostate cancer. Patient characteristics are shown in Table 2. Significant differences in post-DRE urinary IL18Bpa levels (normalized by total protein) were found between cases with and without cancer on biopsy ($P = 0.018$) (Fig 7*A*). The area under the receiver operating characteristics curve was 0.658 for IL18Bp/TP (95% CI (0.546 - 0.769, $P = 0.011$) versus 0.502 for PSA (95% CI 0.3741- 0.630, p=0.975) in this cohort (Fig 7*B*). The sensitivity for cancer detection was 69% and the specificity was 56%. In contrast, there was no significant difference in serum IL18Bpa levels between normal cases (n=10) and cases with prostate cancer (n=69), but IL18Bpa levels in serum from cases with high Gleason sum (7 or more) were significantly elevated compared to those of low Gleason

score (6 or less) (P = 0.029) (Fig 7*C*). Serum IL18 levels were also measured, but levels of this cytokine did not correlate with cancer status or Gleason score (Fig 7*C).*

Discussion

In this study, we demonstrate for the first time that prostate cancer contains IL18BPa and that the prostate cancer cell lines PC3 and DU145 secrete a bioactive form of this cytokine. In addition, the IL18 inhibitor IL18BPa skewed the *in vitro* human immune profile, and was expressed in prostate cancer cells as well as in macrophages in radical prostatectomy specimens. Significant differences in post-DRE urinary IL18Bpa levels were found between cases with and without cancer on biopsy, and serum IL18Bpa levels correlated with Gleason score.

IL18Bp is a member of the Ig superfamily and resembles the extracellular segment of cytokine receptors (but distinct from the IL1 and IL18 receptor family) (6). Human IL18Bp has 4 isoforms, a, b, c and d, derived from mRNA splice variants (11). Isoform a, the most abundant isoform, exhibits the greatest affinity for IL18, while type c has 10-fold weaker affinity (6, 11). In contrast, isoforms b and d lack the ability to neutralize IL18. IL18Bp has N glycosylation sites, and band sizes of isoforms a, b, c and d on Western blot are 42kDa, 16kDa, 40kDa and 35kDa, respectively (12). PC3 and DU145 each showed a single band on Western blot at approximately 42kDa; therefore the main IL18Bp isoform found in prostate cancer cell lines appears to be isotype a (IL18BPa).

Recent findings in mice and humans support the idea of cancer immunoediting (13). In the so-called elimination phase (cancer immune surveillance), NKT cells, NK cells, γδ T cells, T_H1 cells and $CD8^+$ T cells destroy tumor cells by producing IFN-γ. In the equilibrium phase (cancer persistence), tumor cells and immune cells enter into a dynamic equilibrium that keeps tumor expansion in check. Finally, in the escape phase (tumor progression), tumors display reduced immunogenicity and/or engage various immunosuppressive mechanisms in order to attenuate antitumor immune responses, leading to progressive tumor growth (5). Examples of immunosuppressive mechanisms include: 1) Imbalances of T_H1/T_H2 profiles toward T_H2 which induces immune suppressive cytokines such as IL-4, IL-6 and IL-10 (3, 14), 2) TGF-β secreted from tumors promotes generation of Tregs, suppresses CTL proliferation, perforin and IFN-γ expression, and exocytosis of granules, and also suppresses cytokine production and NK cell cytotoxicity (15), 3) Indoleamine 2,3-dioxygenase from tumors also suppresses CTL and enhances Treg-mediated immunosuppression (16).

We suggest that the secretion of IL18Bpa by prostate cancer dampens the anti-tumor effect of immune surveillance. IFN-γ is produced by NK cells, NKT cells and T cells by the stimulation of IL-12 and IL18; the anti-tumor effect of IFN-γ has been observed in many animal models (17, 18). IFN-γ can exert both direct anti-proliferative and anti-metabolic effects on tumor cells, and inhibit angiogenesis within a tumor (19). IFN-γ also activates NK cells and NKT cells against tumors,

enhances MHC class I expression on tumor cells, and stimulates CTL activation and T_H1 cell differentiation (17). IL18Bpa was secreted from prostate cancer cells by IFN-γ stimulation, and the secreted IL18Bpa suppressed IFN-γ expression in $CD8^+$ T cells, $CD8^+CD56^+$ NKT cells and $CD4^+IFN\text{-}\gamma^+$ T_H1 cells, all of which play important roles in immune surveillance. Several poxvirus encode IL18Bp homologous protein, inactivate host-derived IL18 and inhibit NK cell responses to escape host immune surveillance (20). Prostate cancer may also utilize IL18Bp to escape immune surveillances, suggesting that IL18Bpa may be a possible target for cancer treatment. Recently, the use of recombinant human IL18 to treat advanced cancers was studied, and 2/28 patients experienced unconfirmed partial responses (21, 22). The administration of rhIL18 induced IL18Bpa in these patients as well as IFN-γ and GM-CSF. Inhibition of IL18Bpa release might have improved these outcomes.

We found that conditioned media from IL18Bp-transfected PC3 cells suppressed the proliferation of $CD4^+$ and $CD8^+$ cells during incubation with IL2 but without IL18. Banda et al reported that the administration of recombinant murine IL18Bp to the collagen-induced arthritic mouse resulted in decreased proliferation of lymphocytes from spleen and lymph nodes (23), consistent with our data. One possible explanation of the suppression of $CD4^+$ and $CD8^+$ cells by IL18Bp may be that the positive isolation for $CD4^+$ or $CD8^+$ cells may contain a few $CD4^+$ or $CD8^+$ dendritic cells, and IL18 from these dendritic cells was inhibited by the IL18Bpa from

IL18Bp-transfected cells. Elucidating the reasons for the suppression by IL18Bp will require further study. In patients with advanced prostate cancer, the percentage of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells, $CD8^{+}$ T cells and NKT cells from peripheral blood was decreased compared to the normal or patients with non-advanced disease (24, 25). IL18Bpa secretion from prostate cancer may explain these findings.

Serum IL18Bpa levels correlated with prostate cancer Gleason score in our study. Others have previously demonstrated that increased IL18 levels (assessed immunohistochemically) correlated with favorable outcomes in prostate cancer (26). We therefore also measured serum IL18, but found no correlation with cancer status and Gleason score. The data from the Oncomine Cancer TMA Database as well as serum IL18Bpa levels suggest that IL18BPa may be associated with progression of prostate cancer. On the other hand, IL18Bpa in urine after massage was elevated in cases with prostate cancer compared with cases without cancer. IL18Bpa was originally identified from urine, and exists in urine (6). It is known that initial voided urine obtained after DRE is enriched in prostatic proteins (27). Given that our assays were performed after prostatic manipulation, that prostatic fluids from extensive prostate cancer have elevated IL18Bp, that only initial urine was collected as it coursed through the prostate after prostatic examination (“Voided bladder 3” samples, per Meares-Stamey) (27), and that we normalized to total protein in the urine samples (which mostly comes from prostatic sources after a DRE), we surmise that the IL18Bp found in urine after DRE was at least partly if not mostly of prostatic origin. The use of urinary and

serum IL18Bpa levels for prostate cancer detection, progression, and prognostication would require further, larger scale study.

In summary, our findings of elevated IL18Bpa secretion from prostate cancer cells and cell lines, and concomitant suppression of the T_H1 and $CD8^+$ immune response suggest an attempt by prostate cancer to escape immune surveillance. IL18Bpa may be interesting to study further as a prostate cancer marker and therapeutic target.

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Table 1. Profile of top 20 cytokines in prostatic fluids

	Cytokine	ratio Ext/Min	Signal intensity of Ext group		Signal intensity of Min group	
			Average	SD	Average	SD
1	HGF	6.57	118.24	164.5	18	14.9
2	TGF-beta 3	5.08	0.81	1.42	0.16	0.4
3	FGF-6	2.62	2.88	5.15	1.1	1.51
4	IL18BPa	2.58	4.37	6.67	1.69	2.6
5	CNTF	2.49	8.08	8.58	3.24	2.25
6	ICAM-1	2.41	53.34	68.5	22.15	23.09
7	FGF-7	2.34	1.78	2.05	0.76	1.2
8	IL17	2.34	1.74	2.78	0.74	1.15
9	NT-3	2.32	1.79	2.38	0.77	1.31
10	IL12-p70	2.32	4.41	5.92	1.9	1.48
11	MIP-1-beta	2.28	106.3	196.96	46.67	59.8
12	Endoglin	2.26	2.79	3.95	1.23	2.25
13	ICAM-2	2.25	9.26	10.56	4.12	3.7
14	FGF-9	2.21	3.2	4.04	1.45	1.61
15	IGFBP-1	2.17	19.81	47.41	9.14	17.99
16	MPIF-1	2.16	18.46	31.3	8.55	9.36
17	Activin A	2.11	10.69	15.6	5.06	6.39
18	NT-4	2.08	4.33	5.83	2.08	1.43
19	IL-9	2.08	8.35	9.24	4.02	4.52
20	GRO-alpha	2.07	68.1	146.8	32.87	39

Table 2. Patient Characteristics

Characteristics		Urine samples		Serum samples			
		Negative biopsy	Positive biopsy	Normal	CaP		
					All	OC	NOC
No. pts		32	67	10	69	30	39
Median age (range)		62 (40-81)	60 (45-84)	53 (46-66)	61 (47-69)	57.5 (48-66)	62 (47-69)
Median PSA (ng/ml) (range)		5.4 (0.6-11.5)	5.05 (1.7-20.5)	1.15 (0.5-1.9)	5.29 (0.9-27.8)	4.7 (2.9-13.4)	6.5 (0.9-27.8)
Suspicious DRE (%)		18.7	19.4	0	24.3	13.3	32.5
Gleason Score	6	-	43	-	27	17	10
	7	-	21	-	35	13	22
	8	-	1	-	5	0	5
	9	-	2	-	2	0	2

Figure legends

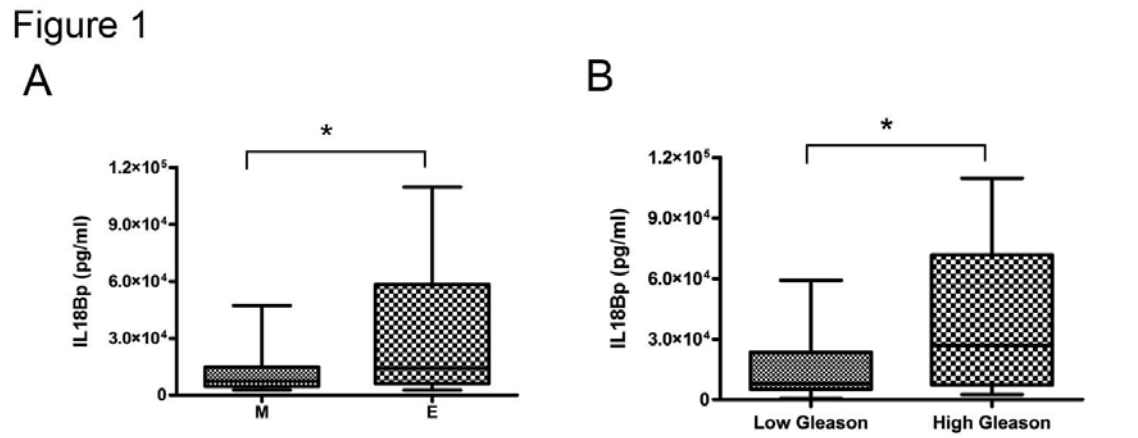


Figure 1. Correlation of IL18Bpa in prostatic fluid with cancer status and Gleason score. IL18Bp levels measured by ELISA were normalized by PSA and analyzed stratified by cancer status (*A*) and Gleason score (*B*). (M: minimal prostate cancer (n=20), E: extensive prostate cancer (n=20), Low Gleason: 6 or less, High Gleason: 7 or more) (*, $P < 0.05$)

Figure 2

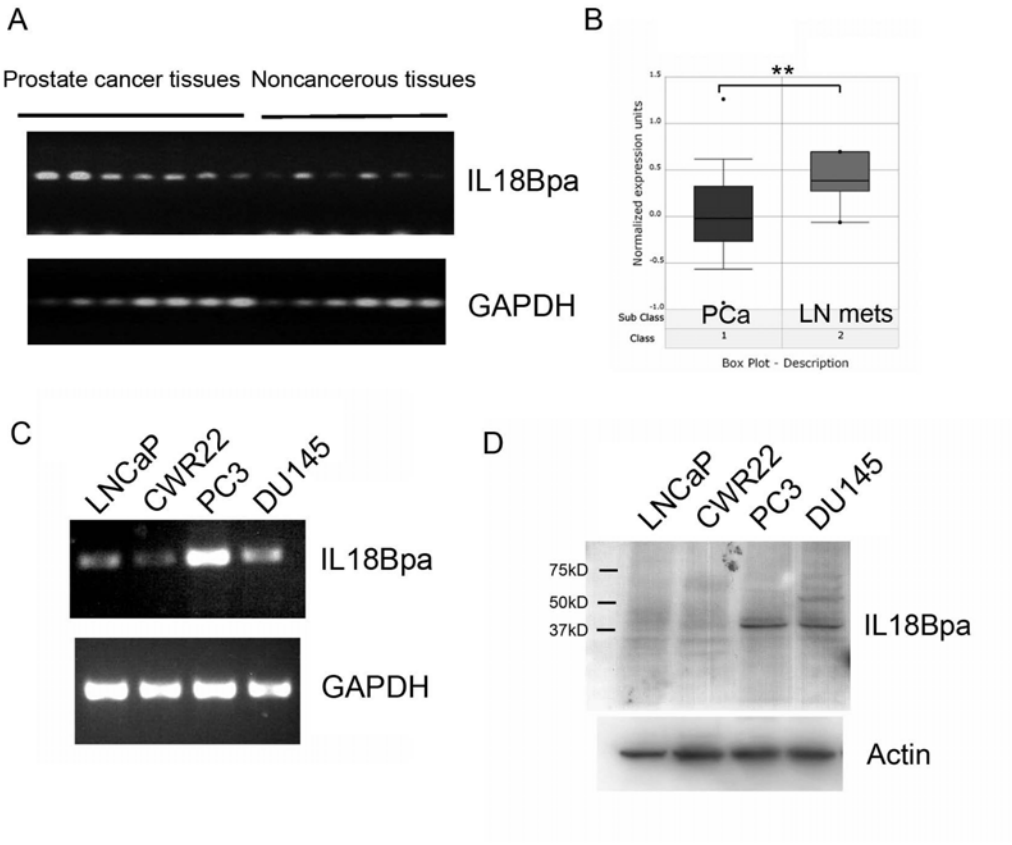


Figure 2. IL18Bpa expression in prostate cancer and cell lines. *A*, RT-PCR analysis showed the expression of IL18Bpa in the frozen tissues of prostate cancer (n=7) and the normal prostate from radical prostatectomy specimens (n=6). *B*, IL18Bpa expression in prostate cancer (n=63) and lymph node metastatic prostate cancer (n=6) from the Oncomine Cancer Microarray Database (http://www.oncomine.org) (**, $P < 0.01$). *C*, RT-PCR analysis of IL18Bpa expression from prostate cancer cell lines. *D*, IL18BPa expression by Western blot for four prostate cancer cell lines. The IL18Bpa band (approximately 42kDa) was detected in both PC3 and DU145 cells but not in LNCaP and CWR22.

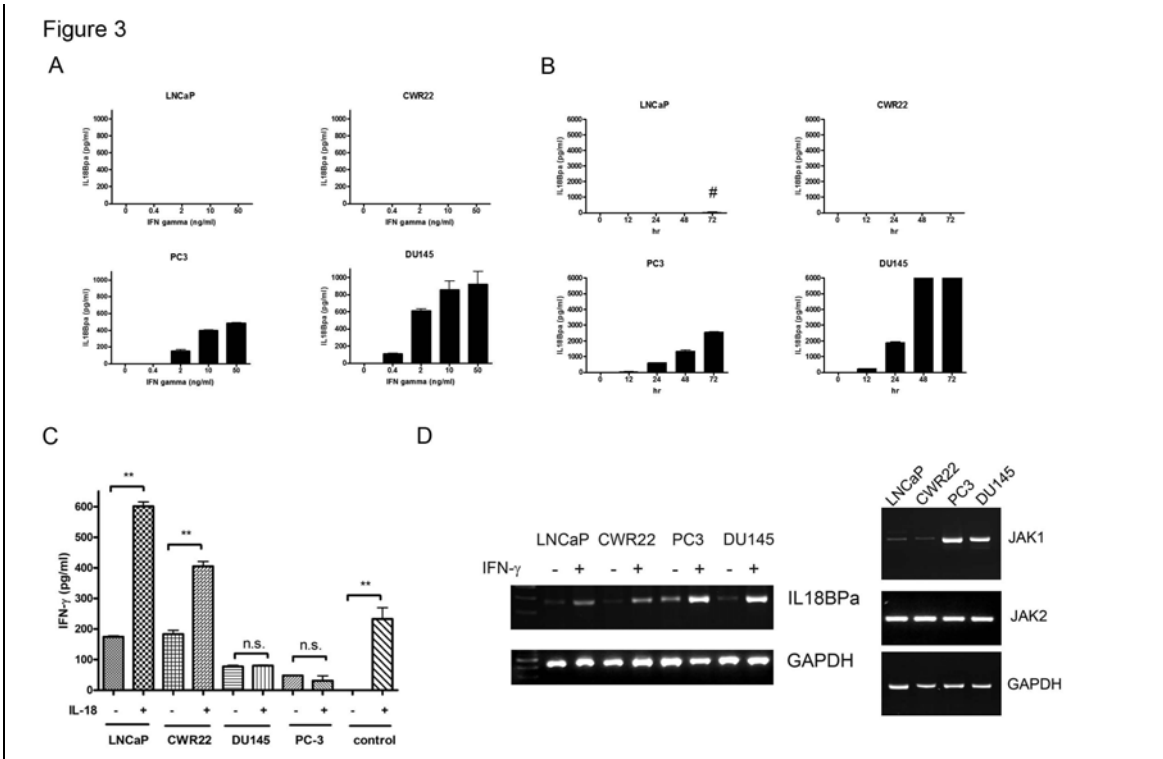
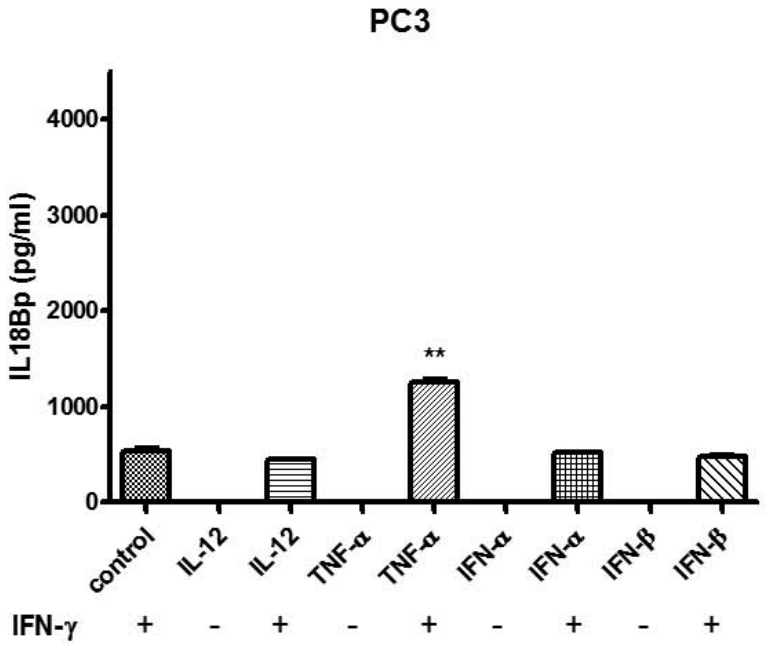


Figure 3. IL18Bpa secretion from prostate cancer cell lines. *A*, IL18Bpa was measured in the supernatants of LNCaP, CWR22, PC3 and DU145 after 24hr-stimulation with IFN-γ (0, 0.4, 2.0, 10, 50 ng/ml) by ELISA (n=3). *C*, IL18Bpa was measured in the supernatants of LNCaP, CWR22, PC3 and DU145 after stimulation with IFN-γ (10 ng/ml) for 0, 12, 24, 48, 72hr by ELISA (n=3). #: trace IL18Bpa (56±7.9 pg/ml) was detected in the supernatant of LNCaP cells 72hrs after stimulation. *C*, IFN-γ-conditioned media from prostate cancer cell lines and control media were used to resuspend KG-1 cells with TNF-α (20 ng/ml) and IL18 (0 or 40 ng/ml). After a 24hr incubation period, IFN-γ production from KG-1 cells was measured by ELISA (n = 3). Means ± SD are shown; **, $P <$ 0.01 (unpaired t-test) *D*, By RT-PCR analysis, IL18Bpa expression was upregulated after the 24hr stimulation with 10 ng/ml IFN-γ, especially in PC3 and DU145 (*right panel*). Whereas JAK2 was

expressed by all cell lines, LNCaP and CWR22 expressed very low levels of JAK1 compared to PC3 and DU145 (*left panel*).

Figure 4

A


PC3
IL18Bp (pg/ml)
4000
3000
2000
1000
0
**
control
IL-12
IL-12
TNF-α
TNF-α
IFN-α
IFN-α
IFN-β
IFN-β
IFN-γ + - + - + - + - +

B

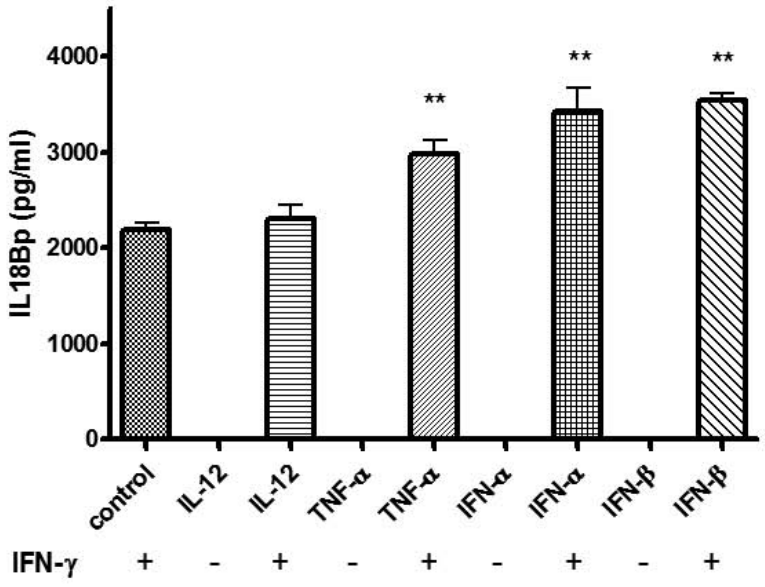

DU145
IL18Bp (pg/ml)
4000
3000
2000
1000
0
**
**
**
control
IL-12
IL-12
TNF-α
TNF-α
IFN-α
IFN-α
IFN-β
IFN-β
IFN-γ + - + - + - + - +

Figure 4. Secretion of IL18Bpa by IFN-γ stimulation can be enhanced by TNF-α, IFN-α and IFN-β. PC3 (*A*) and DU145 (*B*) were incubated with 200ng/ml IL-12, 200ng/ml TNF-α, 10000IU/ml IFN-α or 5000IU/ml IFN-β with or without 10ng/ml IFN-γ for 24hr (n=3), and then IL18Bpa in supernatants were measured by ELISA. (**, $P < 0.01$ compared to control)

Figure 5

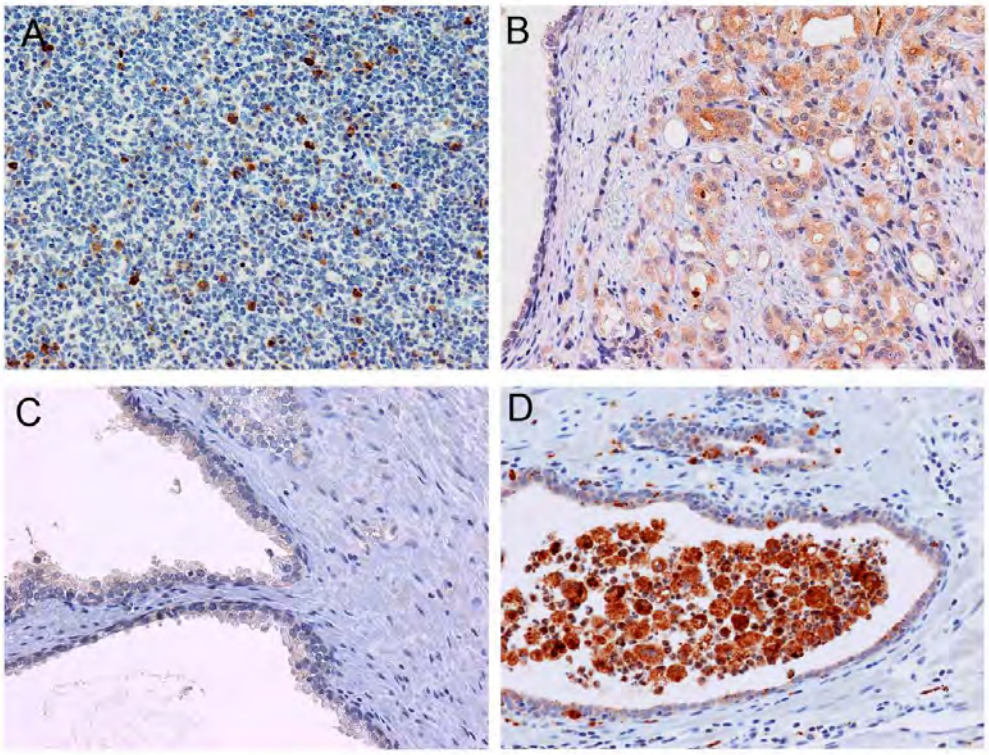


Figure 5. Immunohistochemial analysis of IL18Bpa expression in prostate. *A*, Macrophages in tonsils were stained positively with mouse monoclonal anti-human IL18Bpa antibody (positive control). *B*, Prostate cancer (Gleason score 9) stained positively for IL18Bpa. *C*, Normal glandular epithelium from the same slide as Fig. 5*B* was negative for IL18Bpa. *D*, Glands with inflammation filled with positively-stained macrophages, with positive-staining immune cells also found around the gland and intra-epithelially.

Figure 6

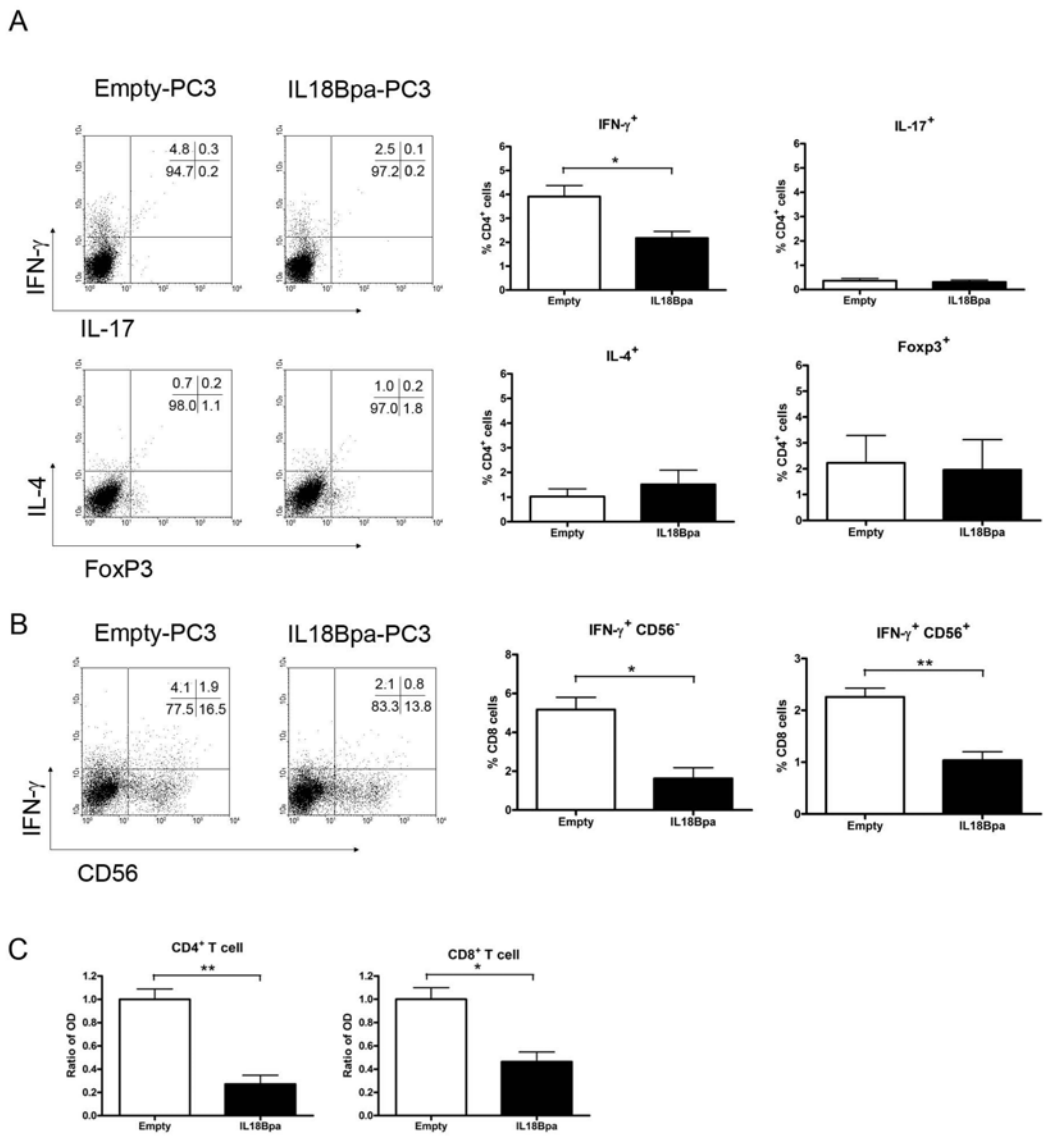


Figure 6. The effect of IL18Bpa on $CD4^+$ and $CD8^+$ cells. $CD4^+$ cells and $CD8^+$ cells isolated from peripheral whole blood of a healthy volunteer were incubated with conditioned media from empty-PC3 or IL18Bpa-PC3 for 3days, and then incubated in media with 20 U/ml IL-2 for 3 days. *A*, Analysis of T_{Helper} subsets: *Left panel* shows the representative results of flow cytometry. The

staining profiles are shown with quadrant statistics. Conditioned media from IL18Bpa-PC3 cells suppressed the T_H1 subset significantly, but did not affect T_H2, Treg and T_H17 subsets (*right panel*) (n = 3). *B*, Analysis of IFN-γ^+CD8^+ cells. *Left panel* shows the representative results of flow cytometry. Conditioned media from IL18Bpa-PC3 suppressed IFN-γ^+CD56^- cells and IFN-γ^+CD56^+ cells significantly (*right panel*) (n = 3). *C*, WST-1 analysis of $CD4^+$ and $CD8^+$ cells after 3 day-incubation with 20 U/ml IL-2. Conditioned media from IL18Bpa-PC3 significantly suppressed $CD4^+$ and $CD8^+$ cells.(*; $P < 0.05$, **; $P < 0.01$)

Figure 7

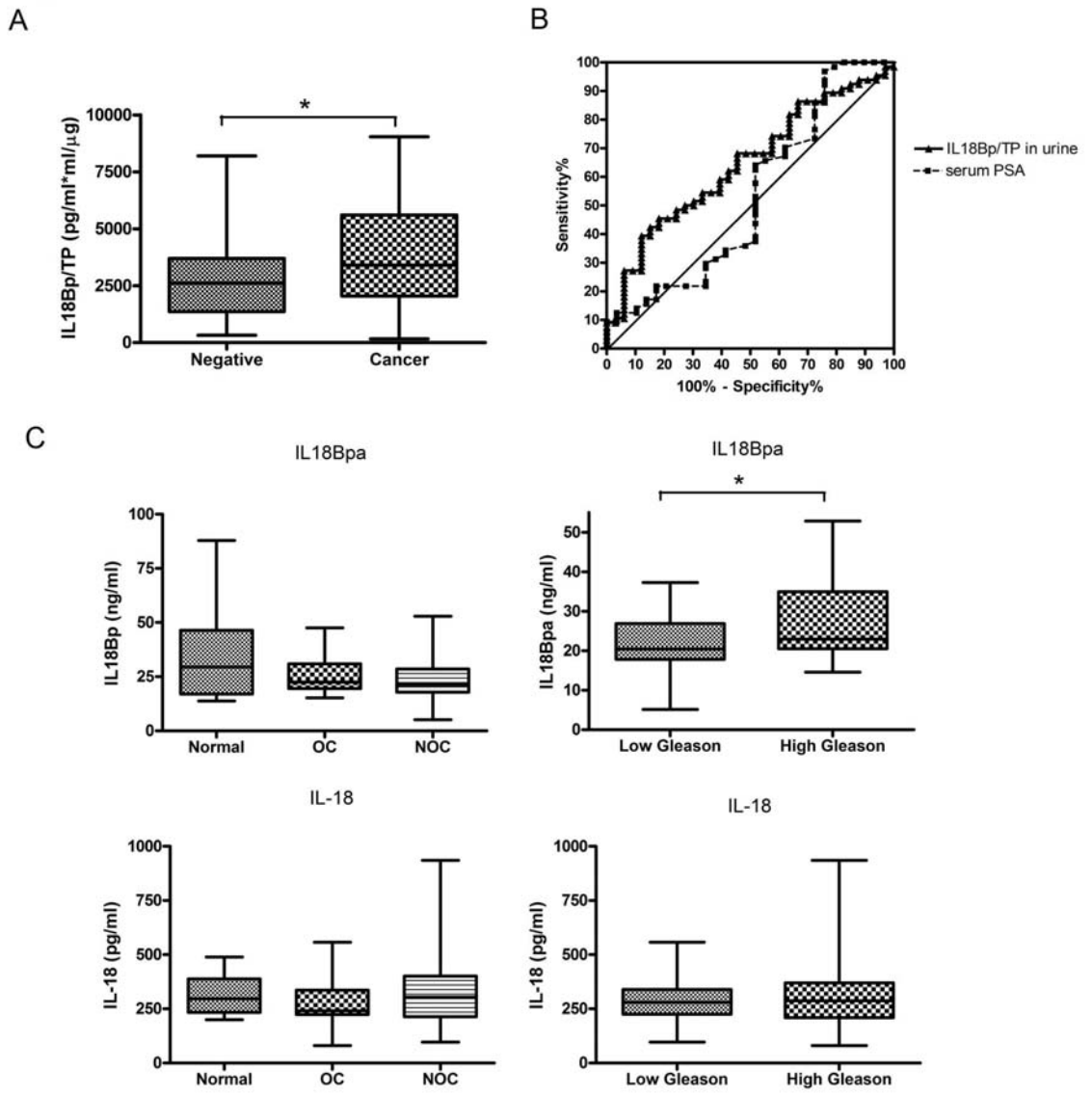


Figure 7. IL18Bpa levels in urine after DRE and in serum. *A*, Significant differences in post-DRE urinary IL18Bpa levels (normalized by total protein) were found between cases with and without cancer on biopsy. *B*, The area under the receiver operating characteristics curve was 0.658 for IL18Bp/TP (95% CI (0.5461 - 0.7692, p=0.011) versus 0.502 for PSA (95% CI 0.374- 0.630, p=0.975) in this cohort. *C*, IL18Bp (*upper panel*) and IL18 (*lower panel*) levels in serum. Only IL18Bpa levels in serum correlated with increased Gleason score. (*; $P < 0.05$)